THE KINETICS OF CELLULAR PROLIFERATION

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THE KINETICS OF
Cellular Proliferation

Edited by
FREDERICK STOHLMAN, JR., M.D.

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Preface

FOR many years there has been a steady accumulation of data on the kinetics of cellular proliferation using such classic technics as transfusion experiments *morphologic examination of tissues determination of mitotic index and estima*tion of the rate of mitosis with stathmokinetic drugs The development of new technics with the increasing availability of isotopes and the feasibility of auto*radiography has not only catalyzed study of this important area but also has* brought with it new problems The rapid accumulation of data with a variety of technics has led to apparent discrepancies in the interpretation of the mechanics of the normal steady state and the method by which the hematopoietic system meets the frequent demand for increased output In view of this the Hematology Study Section of the National Institutes of Health considered that more rapid progress in this important area could be achieved if a conference were held in which investigators could exchange opinions about problems of technic the assumptions implied in the interpretation of data the control of the steady state and the response to perturbations This it was hoped would resolve some prob*lems and more clearly define others*

It was the intent of the sponsors that not only should the Conference provide an opportunity for discussion amongst workers in the area but also that their experience should be made generally available through publication of the proceedings Here qualified investigators have presented the scope limitations pitfalls and even details of various technics The merits of one approach over another are considered Various models for cellular proliferation are proposed and the pro and cons debated Mathematical models are presented for various experimental possibilities and in discussion the usefulness of such models is defined

As might be anticipated in this the atomic age much of the information presented was obtained with radioactive tracers However it has never been more vividly pointed out than in the first session of this Conference that although tracers are important tools one should not discard the older classic technics of morphology etc No stronger argument could be found for the morphologic approach than Dr Bessis classic demonstration of the exchange of iron between reticular cell and normoblast by use of electron photomicrographs which Dr Castle likened to the Mona Lisa

The organizing committee felt that to derive maximum benefit from the Conference the proceedings should be published at the earliest possible date and at *the lowest cost per volume For this ground rules were necessary All manu*scripts were due at the time of the conference two weeks were given for revision of discussion the length of the manuscript permitted should vary with the time *allotted for the presentation at the conference the number of references and* illustrations permitted bore an inverse relation to the length of the text i e by reducing the text one could increase the number of illustrations or references *The Editor was quite fortunate in that all but a few participants adhered to the* rules Particularly gratifying was the promptness of the participants in returning their discussion and the inclusion of appropriate illustrations tables and refer*ences used in the discussion The discussion which occurred has been grouped* according to topics insofar as possible Accordingly where several speakers di cussed various facets of a given problem, the entire discussion has been placed after the final paper on the subject

As Editor I should like to thank the participants for their cooperation and forbearance, and in particular Dr M M Wintrobe, Chairman of the Hematology Study Section, and Dr E P Cronkite, Chairman of the Organizing Committee, for their encouragement and advice I am particularly grateful to Mrs Rita Lyons and Mrs Marie Cole for patient and devoted secretarial help Lastly the prompt publication of this volume would not have been possible but for the cooperation of our publishers, Grune and Stratton, Inc

FREDERICK STOHLMAN, JR

Bethesda, March 1959

Opening Remarks

By M M WINTROBE

IT GIVES me great pleasure to welcome all of you to this conference The State of Utah, *the University of Utah and especially the College of Medicine,* are very pleased to have you here We wish to thank you for coming and owe special thanks to the participants in the conference who have had the always unwelcome task of preparing in advance a manuscript for publication

Thanks are due also to the United States Public Health Service for its generous *support of the conference and for making possible publication of the proceedings* The U S Public Health Service has provided a subsidy for the publication which will make the proceedings available at a low cost

I wish to acknowledge our debt to the subcommittee of the Hematology Study Section whose responsibility it was to plan this meeting The committee consists *of Dr Cronkite as chairman and Drs Castle, London, Stohlman and Tocantins* Thanks are also due to the local committee of which Dr Athens is chairman

I hope that it will be helpful if I state the ground rules of the meeting This is planned as a working conference Invitations to the meeting have been limited to those who have been actually engaged in studies related to the subject of the *conference In addition, a limited number of others who have related interests,* or are responsible for research which has a bearing in one way or another on our topic, have been included We are anxious that there be adequate opportunity for discussion This is the essential reason for limitation of the time of each of the speakers We hope that the speakers will not feel compelled to adhere too closely to their manuscripts but, instead, will talk freely In some instances they may not have time to state everything which is to be found in their manuscripts These details will, we hope, come out in the subsequent discussion In the general discussion those participants in the conference whose names are listed on the *program will be given preference but, at the discretion of the session chairman,* others will be given an opportunity to join the discussion We are naturally anxious to have the discussion as effective as possible At the same time we hope that the remarks made will be as brief and as much to the point as possible

Discussions at a meeting of this kind necessarily are somewhat formal In order *to make possible a freer exchange of ideas than may be accomplished during the* time of scheduled meetings, dinners have been planned for both Monday and Tuesday evenings, at which there will be no program and no speeches During these two evenings it will be possible, we hope, for all those attending the meeting *to come together informally, both before and after as well as during dinner, for* the purpose of discussing problems of mutual interest

Our subject is stated to be the kinetics of cellular proliferation Actually our topic is both broader and narrower than this We are interested particularly in the kinetics of the cells of the blood

Kinetics is defined as that branch of mechanics which treats of the action of forces in producing or changing the motion of bodies We are concerned today not only with the action of forces in producing or changing the motion of cells but we seek to understand their growth rate their rate of liberation from the *sites of their production and growth, and the factors which govern these* We are interested in determining their total mass and their fate We seek this informa tion in quantitative terms We know a good deal about the morphologic aspects of cell growth and we have learned much about the material needed for the formation of cells as well as the chemical changes which the products of their breakdown undergo In quantitative terms however our information is comparatively meager True we have learned much about the erythron its production rate life span and fate The leukocytes however have presented many problems for those who have sought to gain some insight concerning the quantitative aspects of their production and life span The platelets likewise have presented serious problems in this regard These will be brought out in the discussion and I hope some solutions will be suggested

It may be noted that one of the earliest attempts to establish the life span of the leukocyte was that of Minot and Isaacs in 1925 They transfused blood from a patient with chronic lymphocytic leukemia into a patient with lymphosarcoma Unfortunately Dr Minot is not here to participate in this meeting but his worthy successor will serve as chairman of our first session Fortunately many of those who have wrestled with the problem since Minot attempted to find an answer are on hand today This is because systematic studies in this field are actually of recent origin and most of the literature on the subject dates back less than a decade

In the first session it is proposed to lay the background and present the picture of our current knowledge One fact that has become abundantly clear to those working in this field is that there are many pitfalls often unforeseen in studies of the kinetics of cellular proliferation and unfortunately one can criticize some of the studies and conclusions derived from experiments carried out to date

Because it has been found that radioactive phosphorus is incorporated into leukocytes as desoxyribonucleic acid (DNA) only during the intermitotic phase and because it is not exchanged subsequent to cell division DNA synthesis stability and degradation are subjects of the greatest importance to us These will be considered in the second session of this conference Labeled DNA is a key factor in present-day experiments and another session will be devoted to this There are also other labeling technics which have much to offer in seeking answers to our problems and these will be considered also

A conference of this kind would be incomplete without a discussion of the regulation of leukocyte and platelet as well as red cell production and it is hoped to discuss these topics as well Finally it is proposed to explore the difficult and exceedingly important mathematical and statistical considerations which are involved in studies of the kinetics of cellular proliferation

In this regard it is appropriate to call attention to the fact that the participants in our conference include not only the hematologists and biochemists who have now for some time recognized that they have interests in common but also theoretical chemists statisticians mathematicians and even electrical engineers This we feel sure will be valuable for the hematologist I hope that our new associates will find the collaboration at least somewhat rewarding We know that they have methods of approaching a problem which are quite different from ours and these should be refreshing and stimulating and very probably rewarding I wish therefore to bid our new friends a special welcome

THE KINETICS OF CELLULAR PROLIFERATION

Section I. The Use of Classic Morphologic Technics

W. B. Castle, *Chairman*

Anatomic and Physiologic Facts and Hypotheses About Hemopoietic Proliferating Systems

By E P Cronkite T M Fliedner V P Bond and J S Robertson

POSSIBLY the first anatomic fact related to hematology was the firm establishment by Malpighi in the mid 1600 s of the circulation of blood confirming Harvey s earlier concept of the existence of a continuous exchange between tissues *Only since the development of radioisotopes however, has it been possible* to study without disturbance the transfer and exchange rates of molecular substances and their precise distribution in the various body compartments which may be anatomic or chemical Cell proliferation in hematopoietic tissues has been known for decades The formation of blood cells by the marrow was described by Neumann and Bizzozero[19] and mitosis in the bone marrow by Flemming[19] Although the existence of continual hemopoietic cell production has *long been known methods for quantitative study have been slow in develop*ment thus delaying the application of modern compartment theory[37] to the study of the kinetics of cell proliferation In the last decade many precise methods of studying life span production rates size of proliferative and mature *compartments and labeling of single cells have been developed The existence* of a steady state between production and destruction is now quite firmly established Some information is developing on the mechanism of regulation of the steady state proliferation The reason for this publication is to evaluate the present status of knowledge with respect to turnover time rate life span cell behavior fate function and mechanisms regulating the steady state level between production and destruction As the opening discussion this chapter will

1 Summarize the complex and *peculiar circulatory relations between various* anatomic parts of the hemopoietic system and the body at large indicating interchange of cells etc

2 Present a generalized scheme for proliferation indicating the sites at which it is possible to make static and dynamic quantitative measurements

3 Tabulate quantitative estimates of size numbers life span etc as known

The generalized scheme clearly reflects a certain personal bias Following this introduction others will present data to fill in the gaps to revise existing num

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From the Medical Research Center Brookhaven National Laboratory Upton N Y
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bers, or, perhaps, to destroy all or in part the general scheme based partly on facts and thus thoroughly enjoy themselves while living up to an old saying of Charles Darwin "False facts are highly injurious to the progress of science for they often endure long, but false views, if supported by some evidence, do little harm, for everyone takes a salutory pleasure in proving their falseness" O good, Hamilton, Yoffey, Patt, Cronkite[29], Rigas[36], Ottesen[32], and Cronkite[14] have proposed models or schemes for one or more cell lines which we are reluctant to accept *in toto* for various reasons that will almost certainly be brought out in later presentations and discussions

Actually, the premise stated earlier of a simple steady state is a gross over simplification Most tissues are to some degree in a state of flux, their population size varies with the balance between production and removal For example, note the production and disappearance of calluses of the skin and changing red cell mass with increasing altitude However, with constant environment, there is a surprising constancy of the size of the circulating red cell mass, platelet and leukocyte concentrations The fact that a steady state can be maintained, where cell production equals cell loss (removal by senescence and random loss), implies a delicate control mechanism responsive to small changes in some signal The rapid return to the steady state following losses of greater magnitude, such as may be encountered following burns, large hemorrhages, etc , suggests the existence of a second servo mechanism sensitive to rather gross changes in the system involved In fact Stohlman[42] has presented evidence for a dual control of erythropoiesis

The relationship between the various parts of hemopoiesis and the body at large are shown in figure 1 A glance reveals the extreme complexity of the chart which may serve its only practical purpose in demonstrating the complexity of the relationships that participate and interact in the ultimate regulation of hemopoietic balance In some instances one can only measure concentration, and this may be very misleading since the mature and the proliferating compartments under some circumstances can be drastically reduced in size without change in the concentration per unit volume The turnover time will remain constant but turnover rate will be greatly reduced It has been known since Neumann and Bizzozero[19] that red blood cells, certain leukocytes and platelets are made in the bone marrow, enter the venous circulation, become mixed in the lungs, and are distributed to all tissues via the arterial circulation Simultaneously numerous large medium, and small lymphocytes and small numbers of monocytes and plasma cells from the lymph nodes enter the venous circulation via lymphatic channels to be mixed in the lungs and distributed throughout the body Since the concentration in the peripheral blood is relatively constant for each cell type, cells either recirculate are used for some function in the tissues die and are destroyed intravascularly or are removed and destroyed extravascularly, e g , in the reticuloendothelial system (RES) after phagocytosis These problems were in part appreciated late in the last century Metchnikoff, Goldman Ranvier, Aschoff (reviewed in reference 19), and probably others were aware of the migration of cells from one part of the body to another and were concerned about transformation from one cell type to another The awareness and consideration of these problems led to the ideas of storage pools (extra or intravascular) With respect to the lymphocyte, we shall enumerate some of the various ideas of its

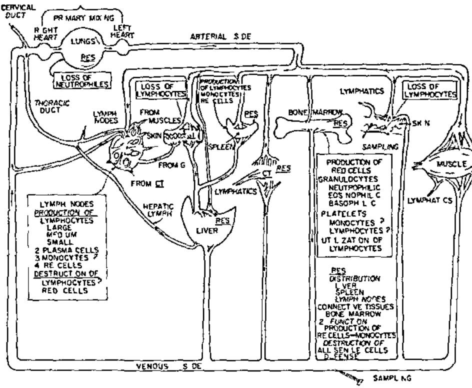

Fig. 1 —Schematic diagram of the relationship between the lymphatic and blood circulation systems indicating sites of production, possible destruction, and the complex interrelations which result in a final hemopoietic balance

function that have developed, some of which may appear puzzling today. Some of these are incorporated into the general scheme in figure 1. The problem is excellently reviewed by Fichtelius[71] and is summarized below.

Lymphocytes have been believed to migrate through the intestine (Bunting and Huston), to recycle from lymph to blood and back (Sjovall), to be filtered out in the bone marrow and serve as precursors of all other blood cells (Jordan and Speidel and championed now by Yoffey)[46], to disintegrate in the blood, to be transformed into bone cells (Okland), to be transformed into epithelial cells (Andrew), and to be filtered out and destroyed in the lungs (Weisberger). Virchow noted that the lymphocyte was active in defense against infections. Maximow and Bloom consider it a stem cell for mononuclear phagocytes, and possibly the neutrophil or other cells under some circumstances. Lymphocytes have been connected with antibody production. Andreasen[2] has ascribed a unique transport role to the lymphocyte of carrying concentrated proteins from one organ to another without influencing the colloidal osmotic pressure of the plasma. The above and many other functions have been ascribed to lymphocytes. It is essential to always recognize that the function of the specific cell cannot be dissociated from its methods of disappearance from the blood and its possible reappearance. These considerations will in turn influence any kinetic analysis of the relations between production and destruction in the steady state or during changes in the steady state. Past investigators have been well aware of the problems and have had to struggle along and interpret a static morphologic picture without benefit of modern technics that permit quantification of the dynamic state

Many functions have been ascribed to monocytes eosinophils and neutrophils Space will not permit discussion of these However a key problem in interpreting observations of the apparent behavior of a cell is whether its mature life is spent exclusively intravascularly or whether a portion of its life span is spent extravascularly with recycling through the circulating blood The red cell and the platelet after extrusion from the marrow normally spend the rest of their lives intravascularly[23, 41]* Evidence for the existence of storage pools is good The storage pool for the red cell is in the marrow The platelet also has a readily available marrow pool[3] and possibly an intravascular storage site (reviewed by Cronkite[29]) There is considerable controversy about the neutrophil Algire has repeatedly seen the neutrophil leave the circulation by direct in vivo microscopy but has never seen it re enter the circulation Osgood[9] postulates a large extravascular pool whereas Craddock and Perry[33] and Hollingsworth and Finch[27, 28] consider the storage pools to be intramedullary (not yet released) and intravascular in areas of sluggish circulation These unresolved problems must be considered in any analysis

A dream of the early hematologist was the labeling of specific cells in order to watch their transformation and fate The only method until recently was staining by vital dyes or by phagocytosis of some visible material thus limiting study to movements of phagocytic cells usually believed to be mature functioning cells such as the neutrophil the histiocyte and the monocytes † The interchange of cells between tissues has long been appreciated but only recently has it been shown that some of the circulating cells are in reality immature cells capable of mitosis and possibly retaining pluripotential capacities The evidence for this is threefold

1 The establishment of cultures of cell lines from normal blood by Ball[6] and the observation of mitosis in culture of human blood[29]

2 The circulation of cells capable of synthesizing DNA and thus destined to divide again separated into five general cytologic characteristics was an observation by Bond et al[11] made possible by a specific DNA precursor and autoradiography

3 Radiation protection studies prove that there is either a single pluripotential cell circulating or multiple cells each capable of re establishing its own cell line in the fatally irradiated animal This has been extensively reviewed by Bond and Cronkite[10]

The concentration of DNA synthesizing cells in the blood is very low generally less than 10 per cu mm in normal individuals[11] However it has been shown that the concentration of DNA synthesizing cells entering the circulation via the lymphatic channels is high of the order of many per cent Labeled cells appear in the lymph within minutes after the injection of H^3 thymidine before they have had time to divide Presumably, they are destined to divide elsewhere We have had the opportunity also of seeing a mitotic figure in the blood of a girl with infectious mononucleosis In vitro studies confirm the presence of DNA synthesizing cells in the lymph Presumably, a similar outpouring occurs from the marrow, the spleen, and, perhaps, the thymus It would appear that these DNA synthesizing cells are either destroyed or rapidly distributed to various areas of the body to perform some function, of which proliferation seems to be an integral part Whether these cells have the *capacity to perpetuate themselves* or go through n divisions to multiply at a site of need is not clear The radiation protection studies of Swift et al (in reference 10) strongly imply that some of the cells can settle down, perpetuate themselves, and produce all cell lines of the marrow

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* The red cell in abnormal conditions may leak out of the vascular bed into the tissues and be reabsorbed via the lymphatics Under some conditions survival following this extravascular circuit will be reduced[4]

† There is no question about the mature nondividing nature of the neutrophil but there is considerable evidence now that the histiocytic and monocytic cells are rapidly turning over by cell division and use

It appears fair to say that all cell proliferation arises from mitosis Even if it turns out to be correct that lymphocytes are trapped in the marrow and then transformed to other cell lines,[19, 21, 29, 45, 46] the lymphocytes still are formed by mitosis in the lymph nodes and may even divide after the postulated trapping in the marrow Irrespective of what the precise mechanism of production may be all mature cells have an organized, identifiable tissue of origin in which mitosis is observable In establishing these cellular factories admittedly complex upon observation, nature has introduced certain cytologic differences that enable one to unfold the complex solid geometric bodies into a linear flow consisting of successive compartments from the earliest identifiable stage to cell death each believed to be flowing into the next in conformance with steady state principles

In this introductory paper, the various broad compartments that probably exist are listed, and then the first three compartments are subdivided These compartments are

I Primitive Progenitor Pool
II Multiplication and Maturation This may have multiple steps consisting of successive multiplicative mitoses
III Maturation and Storage
IV Peripheral Blood
V Extramedullary Pool
    Intravascular vs Extravascular
VI Fate—Removal
    Senescence or Damage, with Removal by the RES
    Functional Use
    External Loss

In figure 2 is shown a flow sheet for the first three general compartments and subdivisions The production of cells can be studied in diverse ways One can enumerate the ratio of one cytologic type to another, which has been done classically and is reviewed[8, 38, 44] The ratio of cells in mitosis can be enumerated[25, 30, 39] One can label cells that are in desoxyribonucleic acid (DNA) synthesis preparatory to mitosis The latter method is suitable for autoradiography[11, 15, 16] The flow of cells from lymphatic channels, combined with bookkeeping, has long been used[45] Various labeling methods permit a study of the disappearance or life span of the mature cell, e g, N^{15} glycine for the red cell[40] For completeness the still valuable Ashby technic[5] of differential agglutination for determination of survival time of transfused red cells must be included Thus, the combined classical and new technics now available make it possible to study practically every compartment and subdivision of the general model, providing that *the technic itself does not disturb the normal state.* It scarcely needs to be emphasized that certain stages of the mitotic cycle are exquisitely sensitive to ionizing radiations The flow rate from one compartment to another, with specific obvious exceptions, could not be adequately studied prior to the development of permanent DNA labels suitable for autoradiography, such as radioisotopes of thymidine (specific DNA precursor) or other nonspecific substances which in part are incorporated into DNA With these labels, cells are marked from DNA synthesis to death and disposal The label is diminished by one half at each cell division Thus, the time for appearance of mature cells, their subsequent behavior, and time for dilution of the label in each compartment can be measured, and from these measurements the flow rates can be calculated, as Bond's later article will demonstrate

Our general model indicates the necessity for the perpetuation of a primitive cell type, an obvious fact commented on long ago by Cowdry[12a] and probably well appreciated by cytologists soon after the continual production of blood cells was fully recognized The full implications with variations in the scheme have been expanded by Osgood[29] The possibility of doubling divisions by cells which have lost the capacity to perpetuate their own cell line exists In our scheme (fig 2), with successive doublings the flow rate from one stage to another in compartment II, the multiplication compartment, must increase in the ratio $1:2:4 \cdots 2^n$ The flow rate (K) is $N/t_G = K$, where N = number of cells in the compartment, t_G = generation time If t_G is constant for each compartment, the ratio of identifiable cells for each compartment must be $1:1:2:4 \cdots 2^n$ How

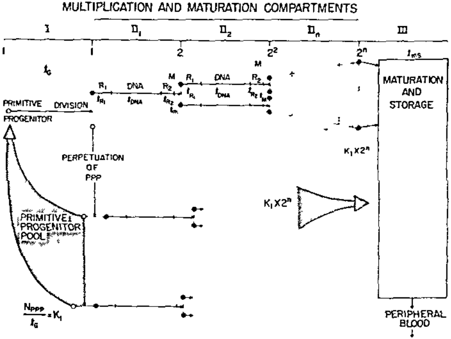

FIG. 2 —Scheme for hemopoietic cell proliferation, indicating perpetuation of a primitive pool, multiplication and division of the generative cycle into its various components

ever, with a variable t_G there will be divergences from this ratio In fact, our data and Baserga's data[8] closely approximate the above ratio, whereas others diverge seriously Divergences may also result from inexplicable losses or gains in cells Yoffey's concept[29] could explain gains We suspect that most divergences are on the basis of technical differences and variable generation times

Sainte Marie and Leblond[33] have worked out a stem cell renewal scheme for lymphocytes in the rat thymus, involving a flow from the reticular cell to the *small lymphocyte, that appears compatible with our generalized scheme*

The essential ingredient of cell renewal is mitosis The generative cycle consists *of four successive stages, a postmitotic rest (R_1), the period of DNA synthesis (DNA), premitotic rest (R_2), and mitosis The sum is the generation time* Each interval has a different mean time, the distribution of these times is not clear Thus, the distribution of generation times is not established The percentage of DNA labeled cells determines approximately the ratio of DNA synthesis time to generation time (t_G) The percentage in mitosis determines the ratio of mitotic time (t_M) to generation time (t_G) By successive sampling, one can study the appearance of label in the mitotic figures and ultimately in mature, functioning cells, as has been done previously,[15, 16] and certain numbers can be fixed to the time for the various stages of the generative cycle A method better than simple bookkeeping is a kinetic study based on a simple compartment theory of steady state systems, as will be presented later by Bond

In recent months we have had the occasion to re evaluate the mitotic index of human bone marrow [5] In the analysis of the data it has been necessary to assume that the times in each multiplying stage *from the primitive precursor through the last division of a definitive cell line, are equal The mitotic index* based on 7 normal males and 2 females is 6 93 per 1000 nucleated cells, based on a minimum enumeration of 5000 cells From this, the preceding assumption and the ratios of various cell types, the mitotic index for the red cell series is estimated as 44 8/1000 cells capable of division Similar calculations reveal an index of 20 2/1000 dividing cells for the granulocytic series These are very similar to those in Rohr[38] and somewhat less than Japa's[30] which were based on 3 individuals and only a 1000-cell count

It is feasible to observe the appearance of label in mitotic figures after serial bone marrow aspirations In principle, as shown,[15] one can derive estimates for the sum of premitotic rest mitotic and DNA synthesis time and their distribution, *from such observations on a single cell population In the study of the bone marrow, the problem is compounded by the presence of at least three cell populations* marching through our scheme at different rates So far, our technic is not adequate for the precise cytologic identification of the cells that are observed in mitosis Perhaps we can later sort out and assign the labeled mitoses to the right cell lines

It may be useful to make some simple calculations to bracket the average turnover (generation time) in the marrow, based upon our own estimates of mitotic index in both the red cell and the granulocytic series From our mitotic index for the granulocytic series we can compute a bracket for turnover time (generation time) of the granulocytic series, as follows *The generation time (t_G) on the average equals the mitotic time (t_M) divided by the mitotic index (M),* or $t_G = (t_M/M)$. Mitotic time has been estimated to be between 0.5 and 1.5 hours. Taking these as a minimum and a maximum, one can estimate

$$t_G = \frac{0.5}{0.0202} = 24.7 \text{ hr for } t_M = 0.5 \text{ hr}$$

$$t_G = \frac{1.5}{0.0202} = 74.3 \text{ hr for } t_M = 1.5 \text{ hr}$$

If our mitotic index is correct, the average generation time of the myeloid precursor should be between these values. Similar computations can be made for the red cell–dividing precursors, using our values given earlier:

$$t_G = 11.2 \text{ hr for } t_M = 0.5 \text{ hr}$$

$$t_G = 33.5 \text{ hr for } t_M = 1.5 \text{ hr}$$

It is now possible to estimate the size of the polychromatophilic normoblast compartment. Earlier work[15, 16] had shown that polychromatophilic normoblasts label immediately and hence are dividing cells, whereas the orthochromatic normoblasts become labeled only by division of its precursor, the polychromatophilic normoblast. Therefore, there are two times as many orthochromats as polychromats. The following equation expresses a simple steady state:

$$\frac{2N_P}{t_G} = \frac{N_{Rbc}}{t_{Rbc}} \quad \text{i.e., number}$$

formed per day = number dying per day.

N_P = number of polychromatophilic normoblasts
$2N_P$ = number of orthochromats
t_G = generation time = 0.94 days, the midpoint of previous range*
N_{Rbc} = total number of red cells in the blood of a 60 kg man
$= 0.069 \times 60 \times 10^3 \times 10^3 \times 5.0 \times 10^6 = 2.07 \times 10^{13}$
t_{Rbc} = life span of red cells or 120 days

Hence,

$$N_P = \frac{N_{Rb}}{t_{Rbc}} \cdot \frac{t_G}{2} = \frac{2.07 \times 10^{13}}{120} \times \frac{0.94}{2} = 8.1 \times 10^{10}$$

polychromatophilic total or 1.35 × 10^9 per kg. The total erythroid series, assuming an approximation to the 1 2 2^n ratio, will be 5.23 × 10^9 per kg, which is very close to Donohue's value of 5.36 × 10^9 per kg[17, 18] that is based on Suit's total marrow technic[41] and counts of a large sample of human marrow.

So far as the neutrophil is concerned, it can be stated that it takes a minimum of from 36 to 48 hours from H^3 thymidine-labeling of DNA for the last myelocyte to divide, mature, and enter the blood stream as band cells. It takes from 48 to 60 hours for the segmented neutrophil to do the same thing.[15, 16]

Computations similar to those for the red cell can be made for the granulocytic series, making certain rather broad assumptions. Bond's kinetic analysis indicates an upper limit for the average stay in the blood of between 1.6 and 2.3 days for

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* Similar to time obtained by Bond, based on kinetics.

the neutrophil. If we assume that none returns to the circulating blood, the life span for our purposes is this time. The midpoint of our range for generation time for the granulocyte based on mitotic index is 2.06 days, and the mean of Bond's estimate is 1.95 days for the upper limit of average time in blood. Assuming a leukocyte count of 7500 per cu. mm. and a 60 Kg. man, one can estimate again

$$\frac{2N_{PN}}{t_G} = \frac{N_{Neut}}{t_{Neut}}$$

N_{PN} — number of last myelocytes to divide

t_G — generation time — 2.06 days

N_{Neut} = *total number circulating neutrophils*

$= 7.5 \times 10^3 \times 0.069 \times 60 \times 10^3 \times 10^3 \quad 3.1 \times 10^{10}$

t_{Neut} — upper limit of average time for neutrophils in blood = 1.95 days

$$N_{PN} = \frac{N_{Neut} \times t_G}{t_{Neut}} = \frac{3.1 \times 10^{10} \times 2.06}{1.95 \times 2.0} \quad 1.6 \times 10^{10}$$

If one assumes four divisions, the total number of metamyelocytes and less mature cells would be 1.02 × 10^9 per Kg., which is considerably smaller than the estimate of 5.3 × 10^9 per Kg. of Donohue et al.[18] The discrepancy may indicate *that the upper limit for the average time in the blood by Bond et al. is high by a factor of five.* Making this correction, the average time of neutrophils in the blood would be about 10 hours, which is closer but still larger than the estimates of Brecher et al.[12] and of others discussed in reference 29.

The question of the location of the large mass of readily available neutrophils shown to exist in the data of Craddock et al.[13, 33] and Hollingsworth and Finch[27, 28] remains to be answered. The average size of any compartment is proportionate to the time spent in this compartment. Donohue's data[18] indicates approximately *equal numbers of metamyelocytes, bands, and segmented neutrophils in the marrow, totaling about 30 times the number of segmented cells in the circulating blood.* If the true average time spent in the blood is of the order of 10 hours, then the transit time in the maturation and storage compartment in the marrow would be of the order of 300 hours. Our data[15, 16] show a minimum transit time of the order of about 48 hours from the labeling of the myelocyte to the appearance of labeled neutrophils in the blood. It would be a peculiar distribution that has a minimum of 48 hours and an average of 300 hours. In order to rationalize the various observations, *it becomes necessary to reduce the probable average stay in the peripheral blood to around 3 hours,* which would be more consistent with most of the observations on marrow size and our estimate of time in the maturation compartment.

If one accepts Bond's upper limit of 2 days as the average stay in the blood for the neutrophil, and also Osgood's and Platt's[9] estimate of a large extravascular pool of granulocytes, perhaps from 20 to 60 times the number circulating, one would have to invoke a total life span after extrusion from the marrow of from 60 to 180 days. *When one uses the lower estimate for average stay in the blood* of about 3 hours, the average life span after extrusion from the marrow becomes of the order of 2.5 to 7.5 days. In either case, if the extravascular pool were as large as postulated, one would expect to see neutrophils in the extravascular space and this is a rarity according to Brecher[17] whereas marginated intravascular neutrophiles are common (Algire[1] and Brecher[17]) On the basis of the preceding and the data of Craddock et al[13, 22] and Hollingsworth and Finch[77, 78] and the scarcity of extravascular neutrophils one is inclined to doubt the existence of an extravascular pool or if one exists it is probably rather small

If one eliminates the existence of an extravascular pool the readily available neutrophils known to be present somewhere can only be explained by the existence of intramedullary neutrophils and neutrophils marginated on the capillary walls generally or in specific organs The leukophoretic studies of Craddock et al[13, 22] demand a large pool of readily available neutrophils and this must be accounted for between the preceding and/or a markedly accelerated maturation and release These and the earlier problems discussed can not be resolved by making assumptions followed by even logical calculations based only on a few known reliable numbers However as a starting point for further discussions estimates for total neutrophil life span will be made In these estimates the following obvious assumptions are made Possibly other hidden assumptions are not recognized

1 A steady state

2 Equal time in each stage of the multiplication compartment in computing mitotic index

3 No recycling of neutrophils from an extravascular pool or exchange with intravascular marginated leukocytes *

4 Build up in labeled neutrophilic cells in the peripheral blood establishes an upper limit for the average stay in the blood (Bond et al)

For our purposes the total life span of the neutrophil will be designated as the time from the earliest stem cell that feeds the neutrophil series through multiplicative divisions maturation and storage time circulating freely in blood time marginated on capillary walls or extravascular to its death Using this approach the following numbers are obtained (note the qualifying comments)

Stem cell generation time	>2 days
No of and total time for myelocytic division	?
Last myelocytic division	2 days
Maturation and storage	>2 days
Upper limit for average time in blood	2 days
Time marginated on capillaries	?
Time in tissues	?
Total life span	>8 days

How much greater than 8 days is debatable If the progression from the stem cell is orderly like marching troops with few stragglers the deviation around the average will be small Previously[15, 16] we have interpreted the build up in percentage of labeled neutrophils in the peripheral blood as indicating the distribution in times from labeling through terminal myelocytic division maturation and storage Perhaps the build up represents earlier myelocytic divisions as well At any rate, the range in times was from 2 to 8 days giving an estimate of the variability of the system

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* If recycling or exchange occurred to a significant degree one would expect to see the reappearance of heavily labeled cells To date this has not been observed[5, 6]

Another factor that has been introduced into the study of the kinetics of neutrophil life span is the presence of dead cells and the time needed to dispose of dead cells From Brecher et al[19] *it is quite evident that neutrophils can be* disposed of very rapidly In the calculations of Osgood,[29] considerable importance is placed upon the enumeration of apparently dead cells Petrakis et al[34] have looked into the quantification of dead cells and found that in the peripheral blood, smudge forms (presumably dead) in Wright-stained smears are from 3 to 8 times as numerous as stained living cells, using the eosin viability test Thus, he concludes that smudge forms are largely the result of injury to fragile cells and do not necessarily reflect the number of dead cells accurately We have had a similar experience with thoracic duct lymph when comparing intact cells by phase microscopy of wet preparations to the large number of smudge forms on the stained dry smear Furthermore, we see a large number of labeled cells in the marrow that are so smudged that we cannot identify them with certainty[15, 16] We suspect that these represent very immature, possibly pluripotential cells but we cannot prove this point as yet

The preceding discussion and estimates of duration of various stages of neutrophil life are based on similar logic the same or additional assumptions used by others, and very few solidly established facts Accordingly, these and earlier estimates are highly unreliable We can only hope that data will be forthcoming that will establish more solid facts and thus eliminate the need for so many tenuous assumptions now being used by all investigators

Limitations in space will permit nothing more than stating certain generalities about the lymphocyte in the blood The data of Ottesen[37] Osgood[29] Hamilton[29] Cronkite et al,[15, 16] and Gowans[26] are highly suggestive of there being two populations of cells whose probable life spans are measured in a few days and many days, possibly in excess of 100 days in man

We have briefly gone over the probable flow of cells in the proliferating organs, but have not discussed how cells get out of the marrow It is a fact that cells do get out of the marrow, and it is also a fact that the marrow has a closed circulation[24] This and the arterial circulation are illustrated in figure 3 One can imagine that leukocytes crawl out and only have to wait until they are sufficiently mature and get the appropriate peripheral signal However, the red cells appear nonmotile Fliedner et al[23] *have shown that radiation induced atrophy of the* marrow soon results in distention of the sinusoids, their rupture, and intramedullary hemorrhage Perhaps red cells and other cells get out by the reverse process The hemopoietic islands grow until the surrounding sinusoidal endothelium breaks, and then the pulsating effect of the spiral artery milks the cells into the larger sinusoids where they are swept into the general circulation and mixed In addition *Fliedner's unpublished observations on marrow structure strongly indicate* the presence of sphincter like structures that may regulate the flow of blood through the smallest sinusoids thus introducing local changes in pH oxygen tension, and metabolites that may influence significantly cell multiplication and maturation

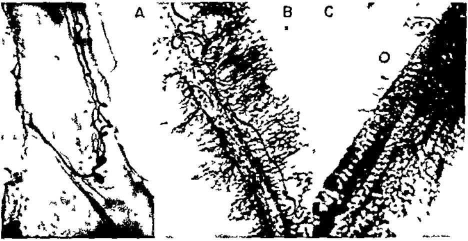

Fig 3 —Injection technic for demonstrating the closed circulation of the bone marrow *A* shows the spiral artery with plastic injection *B* shows the arterial side with filling of the arterial capillaries and partial filling of sinusoids *C* shows more complete filling extending through sinusoidal system into the central venous sinus *B* and *C* India ink injection (from Fliedner T M)

Originally, I had intended to try and summarize all of the data on the various factors discussed in the orderly flow of hemopoietic cell proliferation This turns out to be an impossible task, and all of the allotted space would have been taken up by references alone I suspect that the proponents of each concept that I have ignored will arise and defend their ideas with facts

Summary

1 Cells capable of mitosis are circulating, whether they are pluripotential or directed along one line is not clear

2 These cells are being distributed to the body at large The losses from the lymph nodes will complicate any kinetic analysis of labeling in the nodes Similarly, wherever they may go a kinetic analysis will demonstrate inexplicable gains in numbers

3 The function and fate of a cell cannot be dissociated from a study of the total life span and a kinetic analysis of cell proliferation

4 The flow scheme presented for cell proliferation is probably an oversimplification and is intended only as a starting point for discussion and a general orientation at the level of microscopic anatomy

5 Published estimates on the various stages of the neutrophil life cycle are suspect because of the numerous unproved assumptions that are used in the calculations

6 Marrow circulation is closed, perhaps release from the marrow is a simple mechanical affair

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Discussion

N B Kurnick Dr Cronkite is it possible that there may be substantial variations in the intravascular life span of the granulocyte We repeatedly have observed individuals with marrow hypoplasia secondary to intensive radiation whose peripheral blood counts spontaneously returned to normal over a period of weeks or month even though the marrow remains severely hypoplastic How would this be accomplished if one had a constant release and survival?

E P Cronkite This could be much better discussed after Dr Bond presents the evidence for an upper limit of two days for the mean sojourn in the blood It might be appropriate to mention at this time that if there is a long intravascular life or recirculation then labeled cells would be found for a longer period of time than is observed

H M Patt Our original estimate of a very large exchangeable extravascular pool of granulocytes was an attempt to resolve the apparent inconsistency between the number of circulating cells and the long time span from DNA P^{32} studies [1] We have re-examined this general problem and have concluded from our own data for the dog that the time spent in blood is a matter of hours rather than days On this basis it would appear that there are only a few times as many cells extra or intravascularly as are detectable in blood by conventional methods, this of course refers only to the number of exchangeable cells in the peripheral pool

E P Cronkite Do you still consider the life span in the peripheral blood to be three hours? Is this based on studies with fluorescent labeling?

H M Patt Our estimates of time spent in blood are not based on a fluorescent technic, but rather on the disappearance of cells labeled in vivo with tritiated thymidine As we pointed out last spring [2] it is possible to approach the actual mean time spent in blood by following the disappearance of relatively homogeneous classes of labeled cells as revealed by grain count This procedure sharpens the distinction between the flux of labeled cells entering and leaving the blood In this way, we estimated a half disappearance time of 15 hours which we now believe may be as little as six hours [3]

If there is a mean time in man of two days in the blood and five days in the marrow as indicated by Dr Cronkite then the distribution of cells between blood and marrow should be in this proportion Have you examined your computations on this basis?

E P Cronkite I think we underestimated the actual number in the total blood but I still do not feel that there can be a significant extravascular pool There may be many cells marginated inside the circulation as many people have suggested Such cells would not be detected with a blood volume and leukocyte count

L D Hamilton Would Dr Cronkite estimate the total number of polymorphonuclear leukocytes (PMN) in the body and in the marrow, and their daily production? Your elegant data should enable you to calculate the over all survival time of myeloid tissue the time spent in the bone-marrow compartment and in the circulation and using these data and your mitotic index you should be able to calculate the total PMN s in the body We could then compare this total with the intravascular number to get some idea of the relative size of the extravascular pool

E P Cronkite To do this one must make an assumption as to the number of doubling divisions occurring between primitive progenitor cell and metamyelocyte The last division is the only one subject to direct measurements of time in the compartment rate of maturation and time for appearance in the blood With these values it is possible to estimate a minimum size for the last myelocyte compartment of $1 6 \times 10^{10}$ If there are four divisions prior to that then it is simple arithmetic to add them up However we do not know how many divisions there actually are nor the time for division obviously the size of the compartment in the steady state varies with the time in the compartment I don t think that we have an adequate amount of data at this time to give all the numbers Dr Hamilton requests, but those numbers that are available are presented in the text of my paper together with the implied assumptions

N B Everett Thymidine studies of the life span of the granulocyte in the peripheral blood made by Drs Yoffey, Reinhardt and myself on guinea pigs agree most favorably with those reported by Dr Cronkite for man the only major difference being a slightly earlier appearance of labeled granulocytes in the peripheral blood

J M Yoffey Dr Cronkite how long do you think the mature neutrophil remains in the marrow? Our guinea pig data give a total marrow population of segmented neutrophils of 9×10^8 compared to 4×10^7 in the peripheral blood How do these figures compare with your calculations?

E P Cronkite Not on the basis of calculation but from direct observation the flow of thymidine labeled cells through the marrow into the peripheral blood takes a minimum of two days This time is the sum of the times to complete DNA synthesis postsynthetic rest mitotic time maturation and storage It is the minimum time As the cells build up in the peripheral blood one obtains an estimate of the distribution of these times It appears that the range of the above times is two to eight days with an estimated average time after the last myelocyte division of approximately five days If these observations held true for the guinea pig then from Dr Yoffey s figures of a marrow population 22 times that of the peripheral blood one would calculate a peripheral blood life span of about six hours

G Brecher Dr Cronkite are your computations of total white cell turnover based as are Dr Osgood s on the life span of the erythrocyte?

E P Cronkite It is based on a life span of 120 days for the erythrocyte the total number of circulating mature erythrocytes and the mitotic index The mean time obtained from these considerations is approximately equal to that which Dr Bond calculated from the kinetic analysis I don t think there are any assumptions up to that point The assumptions are that the time in the various compartments are equal i.e, there is a ratio of 1 2 4 2^{n} If that assumption is incorrect figures for total production are certainly incorrect

G Brecher If these calculations are based on the red cell life span then I think one can simplify the problem even further If the number of erythroid mitoses is equal to the number of myeloid mitoses and one accepts your other assumption—namely that for every mitosis you eventually get a cell in the peripheral blood—total red cell production must equal white cell production From your first formula the life span of the white cell and the red cell must be directly proportional to the total number in the peripheral blood From the known relationship of 1 000 times as many red cells as white cell and the 120-day life span of red cells one gets a life span for the white cells of 120/1000 days which is one eighth of a day or three hours

J Ambrus Dr Cronkite referred to several experiments indicating that granulocytes may leave the blood stream but do not re enter We have done some transillumination studies on living lungs We find some large cells which seem to crawl out from capillaries crawl on top of alveoli at times enter alveoli We also have seen these cells re-enter the capillaries These cells are hard to identify and I think they are some sort of macrophage rather than granulocytes Working with large numbers of labeled granulocytes we see these cells migrate into the alveoli However stimuli which will cause pouring out of granulocytes from the pulmonary reservoir (e g epinephrine infection) will not deplete the number of labeled cells in the alveoli proper Hence we feel that this release comes from cells marginated in the pulmonary capillary bed and not from those which have already migrated into the alveoli The latter ascend the tracheobronchial tree and then are swallowed and removed by the gastrointestinal tract We are still puzzled however by the identity of those cells which seem to leave and re-enter and currently are trying to identify them

E E Osgood I should like to emphasize Dr Brecher s point If you postulate the number of cells in the marrow and the rate of cell division as Dr Cronkite has you must have either a very short life span or a large extravascular pool or a pool on the wall of the capillary If there is no extravascular pool how does Dr Cronkite explain the observations of Dr Andreasen[4] that there are large numbers of lymphocytes in such nonvascular tissue as epidermis?

E P Cronkite I don t see how Dr Andreasen s observations on the lymphocytes in the epithelium of the skin and the gastrointestinal tract bear on the neutrophil

E E Osgood Of course it doesn t relate to the neutrophil but it does relate to the fact that there are extravascular extra blood forming tissue pools of hemic cells So also with the neutrophil the number of cells must be directly proportional to the time spent in the compartment after the last division Most of us would agree that the segmented neutrophil doesn t divide and the evidence is that the life span is much longer than three hours As Dr Brecher pointed out, the life span would have to be very short to get the values you present

C G CRADDOCK I fail to see the great conflict The data referred to by Dr Ambrus data from our group and perhaps others, indicate that the bulk of granulocytes do not re enter the circulation once the cells go into the tissues However, there must be a large extravascular mass of granulocytes How long these cells survive after entering various tissues is undoubtedly quite variable We don t know the life span of cells after they leave the blood However, I think that there certainly must be a very large extravascular mass which may resolve the apparent conflict

L D HAMILTON In the peripheral blood of a 70 Kg man (7 5 per cent blood volume PMN = 4 5 × 10^3 cu mm) you have 2 3 × 10^{10} polymorphonuclear leukocytes at any one time Assuming a red cell survival of 120 days and using Japa s figures[5] for the mitotic index (3 3 per cent) for both erythroid and myeloid series (and a mitotic distribution between the myeloid and erythoid series 45 55) erythrocyte production = 10 × 10^9 cells per hour and granulocyte production = 8 2 × 10^9 cells per hour From this we calculate the total number of myeloid cells in marrow is 6 15 × 10^{11} and the daily production of PMN s approximately 2 × 10^{11} You can see that these figures imply large numbers of extravascular neutrophils either in the marrow or elsewhere as a result of large daily production itself 10× the peripheral count However, if many cells matured without cell division or if many daughter cells produced at cell division failed to mature and died, these calculations could mislead one

E P CRONKITE We did not use Japa s figures[5] We used our own for mitotic index of the dividing granulocytic series[4] Our figures are five times as great as Japa s, which are based on total nucleated cell count This would modify the number of cells produced by approximately a factor of five The scheme which I have proposed may not be correct However, I think that in making computations there should be reasonably reliable observations and assumptions I should like to emphasize that if one calculates the way we have been discussing and comes out with variable results, the probability is that the assumptions are in error All too frequently, however, assumptions are made that we do not specify as assumptions, and I think it worthwhile that these be clearly defined I would like to re-emphasize that the size of the dividing neutrophil compartment as we have computed it, is not dependent on red cell life span but rather on the upper limit of the average time the neutrophil spends in the peripheral blood as shown by Bond More careful study of Bond s paper, as well as of my own should clarify this point

D L KLINE Would Dr Cronkite comment on how recirculation and/or reutilization of masses of old cells if it occurs, could affect his calculations

E P CRONKITE I don t know whether reutilization takes place or not I am sure that if there is reutilization any computation based on grain counts would be proportionate to the amount of labeled material that is reutilized and the pool in which it is diluted Whether this would be significant or not I don t know

There is no positive evidence that cells do not recirculate nor is there, from the labeling observations in man any definite evidence that they do recirculate We have observed neutrophils with grain counts up to 60 that emerged between the second and fourth day Thereafter, the mean grain count and distribution of grain counts rapidly fell There was no reappearance of the heavily labeled cells It may be that they were diluted in such a large pool that it was impossible to find them but there is no direct evidence for the recirculation of granulocytes

F STOHLMAN, JR Could you give us an estimate for the error of the mitotic index? Also is there likely to be an intravascular pool in the sense that there will be areas of the vascular system cut off from the general circulation where white cells might be sequestered?

E P CRONKITE The mitotic index is a highly reliable procedure when based on a minimum count of 5 000 nucleated cells in the marrow The statistics are presented in a paper by Fliedner et al[6]

I am quite sure that there are marginated leukocytes in the peripheral blood and there may be as many as three to four times those that are actively circulating

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Observations on Proliferation and Death of Lymphocytes

By Erik Andreasen

ESTIMATION of the mitotic activity in a certain organ or tissue is often desirable. The classic technic of counting mitotic figures in the microscopic sections, however, generally provides unreliable data for the mitotic rates in the organ under study. In our own studies on the mitotic activity of the dorsal epidermis of mice the range of the mitotic rates in the same animal was very marked, often amounting to several hundred per cent. An irregular and apparently capricious distribution of dividing cells was also observed in sections of lymphoid tissue. Substantial variation was seen within the same tissue as well as in lymphoid tissue obtained from different areas. Actually, this phenomenon is not amazing, as we know that the tissues on the outer and inner surfaces of the body are exposed to different, often strictly localized influences. In the case of the inner organs, for example, it is well known that normally at any given moment only parts of the organ are functioning. In consequence, calculations of mitotic activities should be performed by methods which yield data representative for the entire organ in question. To this end we have devised a method for determination of the mitotic activity in hematopoietic and other tissues. The mitotic figures are counted in suspensions of cell nuclei prepared from the whole organ. The counting includes all phases of mitosis, and differential counting of the mitotic phases is possible.[2,4] This is important, as several factors may display a selective effect on any phase of the mitotic cycle.

It is well established that desoxyribonucleic acid forms the main constituent of the nucleus and that formation of the different parts of the cell nucleus requires DNA synthesis. For many years studies have been carried out to elucidate which substances regulate biosynthesis of the various nucleoproteins. Results of previous studies led to the conclusion that the cells in tissue cultures obtain their nitrogen from proteoses. More recent studies, however, have shown that various amino acids also have a definite stimulating growth effect on cells cultivated in dialyzed material. In Copenhagen, where studies of this kind are going on, it has been found by Kieler[7] that glutamic and aspartic acid, arginine, histidine and lysine have a considerable stimulating effect on cell division, methionine and tryptophane have a moderate promoting effect on the mitotic action, while cystine and proline are without any definite effect. Besides, it was found that arginine lengthens the duration of the prophase, while lysine has just the opposite effect. And the stimulation of the mitotic activity caused by aspartic acid and by tryptophane is followed by a disturbance of the cellular development from metaphase to ana-telophase. It was also observed that the mere presence of the necessary amino acids is not enough to secure normal mitosis. The relative concentrations of the various amino acids are equally important.

In my own studies on the mitotic activity in the various lymphoid organs I have been surprised to observe that under certain conditions the lymphoid tissue

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was characterized by high mitotic rates whereas under other conditions the same tissue was marked by just the opposite process namely degeneration of lymphocytes combined with phagocytotic activity carried out by the reticulum Great numbers of mitotic figures in the cortex of the thymus are observed normally but under the influence of stress and metabolic disturbances of various kinds the very same tissue is dominated by phagocytic activity In the cortex of the thymus the balance between cell division or DNA synthesis and cell destruction or DNA decomposition easily is displaced in favor of one of these two possibilities

A high mitotic activity is observed in the lymph nodes especially in the secondary nodules of the cortex under certain conditions however these nodules are transformed into typical centers of phagocytosis and at times they show indications of both mitotic activity and phagocytosis Mitotic figures may be seen in the medulla of the lymph nodes the spleen and in diffuse lymphoid tissues but pyknotic lymphocytes and debris appear much more often Undoubtedly a significant fraction of the enormous number of lymphocytes produced in the body is disposed of in the same lymphoid tissues where it generates

It should also be mentioned that lymphocytes are always present in the normal epithelial covering of the body In the epidermis of the mouse I found[1] the ratio of lymphocytes to epithelial nuclei varied from 2 0 to 3 8 per cent Andrew and Andrew[3] found in man that the lymphocytes constitute from 1 to 4 per cent of the cells of the stratum germinativum In the intestinal epithelium this ratio is still higher A high percentage of the lymphocytes in the epidermis and in the intestinal epithelium is degenerating In addition during the processes of hyperplasia following cutaneous application of solutions of carcinogenic substances I have observed an increase in the number of lymphocytes in the dermis and the epidermis may be infiltrated by a multitude of lymphocytes The ultimate fate of all epithelial lymphocytes seems to be degeneration

Unfortunately adequate quantitative data of the total body pool of lymphocytes are not available but it can be established that a high percentage of all lymphocytes appear to be in a state of degeneration Since the two processes of proliferation and destruction of lymphocytes are found in all lymphoid tissues and dying lymphocytes are present in many types of cellular proliferation the question occurs as to whether lymphocyte segments or split products could be used by the dividing cells for the synthesis of DNA A reutilization hypothesis of this kind is supported by Hamilton s studies[5] [6] with isotopic tracers Histologic evidence is mentioned in this report Trowell[8] demonstrated in in vitro experiments that the same phagocytic cells which normally eat up dead lymphocytes may themselves differentiate into large lymphocytes

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Discussion

J. Ambrus: *Dr Andreasen, is it not a* fact that you can have pure cells grown in semisynthetic media without a lymphocyte anywhere in the vicinity? *Do you suggest* that lymphocytes must be present if other cells are to undergo mitosis? If so, do you suggest that the lymphocytes contribute ready made DNA, so that the whole theory of DNA duplication thereby falls to the ground? I know this has come up in connection with the *reutilization* of nucleic acid templates, and even there it is open to doubt, but if one is seriously maintaining *this as a general function of the lymphocytes* it does raise some rather fundamental questions.

E. Andreasen: I don't think lymphocytes are *necessary for cell division* but very often when you see cell division you also see lymphocytes.

C. P. Leblond: The release of nuclear fragments from pyknotic lymphocytes (as an adjuvant to cell division) has been said to occur in thymus. However, in rats that are kept under normal conditions and free from diseases, pyknoses are rare in the thymus, in spite of the very active cell division. On the other hand, lymphocytes are not found in many areas with high mitotic activity, such as growing hair follicles, isthmus region of stomach mucosa, granulosa of growing ovarian follicles. *Hence I* do not believe that there is any relation between mitotic activity and the presence of lymphocytes.

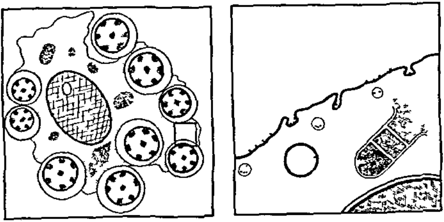

FIG 3.—(A) Erythroblastic islet reticular cell surrounded by erythroblasts The reticular cell packed with ferruginous granules is situated in the center of the erythroblasts which completely surround it

(B) Enlargement of the preceding figure (3A) penetration by the ferritin molecules Iron in the mitochondria One can see the ferritin molecules of the reticular cell penetrating the cytoplasm of the erythroblast (by ropheocytosis) and the mitochondria

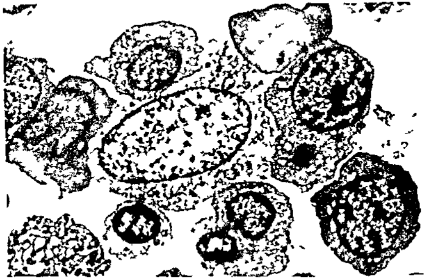

FIG 4.—Erythroblastic islet under lower power magnification in the electron microscope

greatly from this "micropinocytosis" and comprises the following phases (figs 3 4 5–7)

1 The molecules initially adhere to the plasmatic membrane of the cell and cover a relatively large area but always of a submicroscopic order

2 This area is then seemingly drawn in by the cytoplasm and forms an invagination of the order of 500 Å on the whole not very deep

3 By squeezing together the two edges the invagination becomes a vacuole whose membrane retains on its interior surface the particles that were attached to the plasmatic membrane prior to invagination

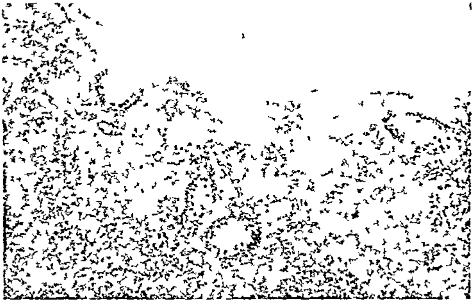

FIG 5 —Typical example of ropheocytosis The granules adhering to the membrane are sucked into a cavity which closes up immediately afterward

4 At a later stage the membrane of the vacuole gradually vanishes its liquid content is absorbed and disappears the molecules are thus imprisoned within the fundamental cytoplasmic matrix These molecules are found solely in the inferior portion of the vacuole formed in this manner The upper section formed by the union of the two sides of this primitive invagination remains bare This aspect is very characteristic The process thus differs from that of pinocytosis in which an absorption of liquid is realized and the cell appears to drink In defining the process described above Policard and Bessis[11] have proposed the term 'ropheocytosis

The phenomenon of ropheocytosis is constant in the normal state and in several diseases There is *absolutely no doubt that it exists Nevertheless it is not* yet known whether all the iron needed for the formation of hemoglobin is introduced by this means or whether a certain proportion penetrates the cells by a *direct route, emanating from the plasma transferrin Moreover it may be as*sumed that several mechanisms exist simultaneously

1 Iron originating from the destruction of aged red cells proceeds directly to the erythroblasts which form the erythroblastic islet

2 Transferrin carries iron to the reticular cells of the bone marrow where it is transformed into ferritin and distributed to the islet erythroblasts

3 *A proportion of iron is delivered to the erythroblasts by the transferrin Several experiments in vitro have shown that erythroblasts are able to extract* iron from transferrin[7-10]

3 Fate of the Erythroblastic Ferritin, Probable Role of the Mitochondria

It has been established that, immediately following penetration into normal erythroblasts ferritin molecules frequently become concentrated into clusters of varying sizes (fig. 6) It is unknown by what mechanism these clusters are formed

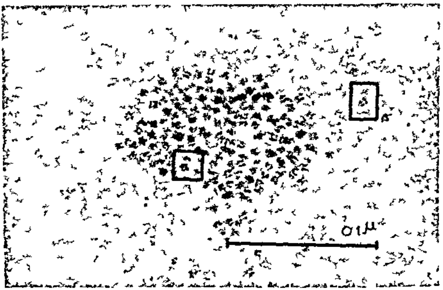

FIG 6 —Clusters of ferritin granules in an erythroblast In each of the two insets one can perfectly distinguish the ultrastructure of the ferritin molecule composed of four ferruginous nuclei Each one has a diameter of 15 Å

In certain cases they may attain a diameter of 0.5 μ Popheocytosis in fact gives rise originally to small vacuoles containing little more than ten molecules per section

These large clusters are very easily seen in the optical microscope and the presence of iron may be demonstrated by Perls reaction They are called sideroblasts The electron microscope has shown that all erythroblasts are sideroblasts Using the optical microscope about 30 per cent of erythroblasts appear to be sideroblasts because only those cells with clusters greater than 0.2 μ in diameter are counted

We do not have a clear understanding of the ultimate fate of either the ferritin granulations or the clusters in the bone marrow of normal individuals All that one can see is the gradual disappearance of the ferritin molecule as cell maturation progresses and hemoglobin appears However a phenomeon frequently encountered in certain pathologic states is the proof that ferritin is present in the interior of the mitochondria The importance of mitochondria in the storage of enzymes is well known On the other hand Rimington[13] has recently drawn attention to the fact that a suspension of liver mitochondria permits certain stages in hemoglobin biosynthesis to occur in vitro The very fact that ferritin mitochondria are encountered is an argument in favor of their role in the series of reactions which commencing with ferritin eventually lead to the incorporation of iron in the hemoglobin molecule In addition ferritin undergoes a singular transformation inside the mitochondria about which we will speak later When the red cell matures at the reticulocyte stage one sees the mitochondria burst diffusing their contents in the surrounding medium after which they disappear

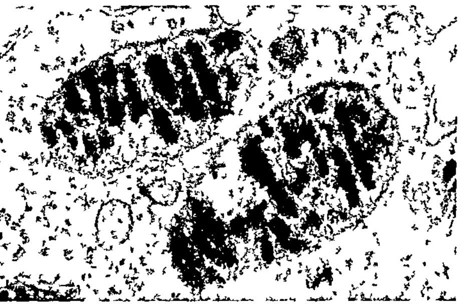

Fig 7—*Aspect of ferritin and ferruginous micelles in the mitochondria Top* one can see granules of dispersed ferritin between the crests of the mitochondria these particles are difficult to see owing to a mixture of ferritin with ferruginous micelles

4 PRESENCE OF FERRITIN IN THE RED CELLS OF THE HYPOCHROMIC HYPERSIDEREMIC ANEMIAS

In thalassemia major the quantity of iron present in the bone marrow shows a definite increase In the reticular cells, hemosiderin is often in the crystalline state in the form of large deposits The erythroblasts are packed with iron either in a dispersed form or as ferritin clusters In nearly all polychromatophilic and acidophilic erythroblasts *ferritin molecules can be seen in the mitrochondria* (fig 7) Their mode of penetration and concentration here is as yet unknown Inside the mitochondria one can study the transformation of the ferritin molecules They lose their characteristic structure and assume a powdery aspect for which we have proposed the name ferruginous micelles' (fig 7) The mitochondria swell and finally burst liberating *the ferruginous micelles into the cytoplasm In reticulocytes and even in erythrocytes, the amount of hemoglobin formed re*mains low in comparison to normal values It seems that the iron may be so obstructed that it cannot be incorporated in the hemoglobin molecule

In addition to the thalassemias other hypochromic hypersideremic anemias have been distinguished Heilmeyer s sidero achrestic anemia[4] the case of Caroli and Coll[5] One can also classify *under this heading anemia resulting from certain* intoxications such as saturnism[2] (lead poisoning) In this case the electron microscope gives a picture closely resembling that found in thalassemia major The erythrocytes may contain so little hemoglobin that in the electron microscope it becomes difficult to distinguish a red cell On the other hand the number of *ferritin molecules, either as clusters or dispersed is very high*

It is important to note that the bone marrow examination of patients suffering from simple hemochromatosis (without hypochromic anemia) shows a very different picture The accumulation of hemosiderin in the reticular cells is again encountered but the succeeding erythroblasts are perfectly normal Ferritin is present in normal amounts in the erythroblasts and disappears when the erythrocytes mature In simple hemochromatosis the overloading of red cells with ferritin is nonexistent

SUMMARY

1 Reticular cells charged with ferritin and hemosiderin are situated at the center of erythroblastic islets This ferritin may be derived directly from the phagocytosis of aged red cells or may be formed from iron obtained from plasma transferrin

2 By ropheocytosis (a mechanism related to pinocytosis) these molecules enter the erythroblasts

3 In all normal erythroblasts iron is present in the form of ferritin It may be found either in the dispersed state or in the form of clusters When of sufficient size these clusters are visible in the optical microscope they are the granules of the siderocytes

4 Normally iron is rarely found in the mitochondria When present it is in the form of ferritin granules or as ferruginous micelles

5 In thalassemia iron accumulates in great amounts in the erythroblasts and is even found in the erythrocytes either as ferritin clusters or in the dispersed form Occasionally it is present in large quantities in the mitochondria in the form of ferruginous micelles In disorders of hemoglobin synthesis this unused iron accumulates in the hypochromic erythrocytes

6 In other hypochromic hypersideremic anemias in which fetal hemoglobin is not found (sidero achrestic anemia hypersideremic anemia saturnism), similar observations were made

7 It is probable that normally iron metabolism takes place in the mitochondria

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Discussion

L G Lajtha Dr Bessis, are you sure of the direction in which the phenomenon of pinocytosis goes? Does the iron go from the reticular cells to the erythroblast or vice versa? We have some evidence that even after 10 to 15 minutes of contact with a solution of iron all of the normoblasts will contain a large amount of iron but at that time very *few of the reticular cells will contain iron* I would submit that the process of iron uptake is a direct function of the cells taking iron from the surrounding medium without interference by reticular cells and it is possible that the phenomenon which you observe is the deposition of iron from degenerating erythroblasts into reticular cells, cell degeneration being a significant factor in the marrow

M Bessis *Of course we cannot follow the phenomenon with electron microscopy* But the same is true in direct smears of bone marrow You cannot follow the maturation of an erythroblast into an erythrocyte However, no one ever suggests that an erythrocyte matures into an erythroblast There are many arguments against that The same is true for pinocytosis We have many reasons to believe that iron goes *from the reticular cells into the erythroblasts* and not the reverse At the moment I believe that the direction is unquestionable It is from the reticular cell to the erythroblast There is some evidence that some siderocytes in disease can lose the siderocytic granulation in the blood stream but this is another question

F Stohlman Jr Dr Bessis *do you envision* that the reticular cell obtains iron directly from transferrin and that the iron is then transferred to the erythroblast Might not iron go to the nucleated red cell directly, and the reticular cell derive some of its iron from phagocytosis of nucleated cells dying in the marrow?

M Bessis There are two ways by which iron enters the erythroblast—the mechanism that I *demonstrated and another involving transferrin Iron given intravenously is* bound to transferrin We dont know whether it goes into the reticular cell (the nursing cell) where it is converted to ferritin and then transferred to the erythroblast or whether it goes directly into the erythroblast It is evident that the latter sometimes occurs since many people have shown in vitro (in the absence of nursing cells) *that iron goes into erythroblasts* Perhaps with *autoradiographs*, we will get a quantitative answer I feel that either mechanism may occur and the frequency with which one or the other occurs is unknown To answer Dr Stohlman s question specifically we definitely have seen reticular cells engulfing erythroblasts in the bone marrow in pathologic cases but in perfectly normal cases you find erythrophagocytosis *in bone marrow* The *nurse cell can* be the same which *performed erythrophagocytosis* or another one which has received iron from transferrin

F L Alpen We have done autoradiographic studies on marrow after injection with iron It is very clear from these studies as well as from those of Dr Lajtha that when one inject *inorganic* or plasma bound iron this iron goes directly into the erythro blast with no intermediate in the cycle These observations do not rule out the importance of the process that Dr Bessis demonstrated because we have always known the importance of so-called stable iron in the production of red cells the second component of the plasma iron disappearance curve for example is best accounted for on the basis of iron which is stored in this fashion In fact when one gives radio-iron you see labeled reticular cells or macrophages about 12 to 24 hours after the injection of the tracer iron This is usually associated with remnants of what appear to us to be disintegrated early normoblasts We interpret this to mean that a few of the cells that originally incorporated the free iron didn t make the grade and were then ingested by the macrophage cell Then this iron may be reutilized by the process Dr Bessis describes

D NATHAN Dr Alpen s explanation of the two different routes also may explain the delayed uptake of labeled iron into the red cell after eight days when the curve slowly increases from 80 to 90 per cent of the injected dose[1]

F STOHLMAN JR. Dr Alpen s observation of the appearance of iron in the reticular cells 12 to 24 hours after injection of iron could readily be explained by death of labeled cells during the process of production This in turn raises a serious question as to the validity of computations based on the assumption that every cell produced reaches the periphery

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Classical Technics for the Study of the Kinetics of Cellular Proliferation

By C. P. LEBLOND

IN THE MINDS of biologists the word "mitosis" is associated with the concept of growth. It is true that cellular proliferation implies not only the replacement of one cell by two daughter cells through mitosis but also some spatial rearrangement to provide enough room for the new cells. Thus, at sites of division, expansion is required. During embryonic life the result of mitosis is essentially an enlargement of the regions where proliferation takes place, that is, growth. Complex cell migrations may also occur, as in the expansion of the blastopore lip. Again, growth is the result.

In adult mammals, however, body growth is usually slow or absent. Even so, there are many regions of cell proliferation. Some of them are easily accounted for, such as the mitoses in the hair follicle, which ensure the continued elongation of the hair shaft, or the periodic outbursts of mitoses in some ovarian follicles which are the prelude to the swelling and rupture of the follicles with release of the ova. In both these cases, there is a structural addition—longer hair or larger follicles—that is, again growth, but strictly localized.

Finally, mitosis may be found in structures such as thymus, spleen, or bone marrow, which show no enlargement with time in adult animals. Neither the number nor the size of the cells show any change; therefore, cells must be lost by migration or degeneration, so that cell production is balanced by cell loss and the cell population is kept in a steady state. Such a cell population may be referred to as a renewal system.[1]

The first step in investigating cell proliferation is to determine which cells are undergoing mitosis. The next step is to find out at what rate mitoses occur. This rate may be expressed as the *turnover time* of the population, i.e., the time taken for the replacement of a number of cells equal to that in the whole population. It is possible to estimate the frequency of mitotic division and thus obtain the *mitotic index* (ratio of the number of dividing cells over the total number of cells in the population). However, mitotic duration has to be known also if we are to deduce the turnover time from the mitotic index. Unfortunately, information regarding mitotic duration is meager. Variations are large, the extreme case being the epidermis, in which mitotic duration may vary with temperature from one to several hundred hours, as shown below. The *colchicine technic*, which arrests mitoses at metaphase, makes it possible not only to emphasize regions of high mitotic activity but also to give a precise figure for mitotic duration and turnover time.

On the other hand, mitotic counts, even after colchicine, tell us nothing about the fate of the cells following division. Newly formed cells cannot be distinguished

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from their neighbor and we do not know how they leave the tissues nor what their pathway of migration is Radioautography after administration of DNA precursors particularly tritium labeled thymidine reveals the fate of those cells which have divided immediately after injection of the precursor

This paper will examine the use of mitotic counts with or without pretreatment with colchicine Other technics will only be briefly mentioned

METHODS

1 Mitotic Counts

Counts of the percentage of cells undergoing division may be carried out in sections or smears but several precautions are essential though often overlooked

Great care is necessary in identifying mitosis and in determining the cell type to which each dividing cell belongs Some authors who write of mitosis really seem to have counted only metaphases Obviously they will report much lower mitotic indices and shorter mitotic durations than those found by other workers The best and usual procedure is to count all mitotic stages from the earliest recognizable prophases to the latest recognizable telophases This requires establishing definite operational criteria for identifying each stage of mitosis These criteria may differ from cell type to cell type

The identification of cell types is more easily said than done since dividing cells lose not only nuclear characteristics but also some cytoplasmic feature Good histologic technic and experience help in assigning dividing cells to a given population but in some cases the difficulties are insurmountable Another precaution is to have in the ocular of the microscope a discrete precise field outline Once the area of this field has been measured for a given magnification the numbers of resting and dividing cells may be recorded per unit area

In tissues in which the size of dividing nuclei differs from that of resting nuclei there is a danger that the larger type will appear in sections more frequently than the smaller type so that frequency estimates may be inaccurate It is then necessary to apply the Abercrombie correction[2] Unfortunately this correction involves measuring the mean diameter of the nucleus an easy task with resting nuclei but a difficult one with dividing nuclei Even so the correction improves results

Following the example of Stevens Hooper[3] other sources of experimental error must be minimized by performing all experiments at the same time of the day and by taking the same portions of the organs to be studied from each animal

2 Use of Colchicine

When an adequate dose of colchicine is injected approximately 1 mg per kg body weight in many mammals the cells undergoing metaphase will remain at that stage so that the count of the number of mitoses after a period of let us say four hours indicates how many cells have divided during that time It was found that 1 mg of colchicine per kg body weight given in a single subcutaneous dose was as effective as if given intraperitoneally or intravenously and a single injection was more effective than repeated smaller injections[4]

The first and obvious value of colchicine is to show accumulations of mitoses and thus make it easy to detect areas of cellular proliferation By far its greatest value is to show how many cells divide over a certain period of time four hours for instance so that it is possible to calculate the rate of division and therefore the turnover time A comparison of the number of dividing cells at a given time in a nontreated animal with that in a colchicine treated animal makes it possible to calculate mitotic duration

Stevens Hooper[3] has stated three important requirements which must be met for a colchicine experiment to give dependable results These must be tested before each experiment and before an untried sample of colchicine is put into use

1 The drug must have no effect on mitotic stages preceding metaphase This can be tested by comparing prophase counts in treated and untreated animals

2 The time at which the drug takes effect should be known e g if subcutaneous injections are used their effects should be compared with those of intravenous injections

3 The effect of the colchicine should last several times as long as metaphase and longer than the experiment This requirement may be hard to meet in tissues where mitosis is exceptionally slow as in the cold acclimated rats studied by Héroux (see below)

To illustrate the kind of results expected from colchicine Stevens Hooper[3] presented the model shown in figure 1

Many objections have been made to the use of colchicine in a recent review[1] these objections were listed and it was concluded that counts of colchicine-blocked mitoses give a minimum figure for mitotic rate over the period of action of the drug Recent results to be reported below indicate that at least in certain tissues the information provided by colchicine is more precise than generally thought

3 Use of X rays

Mitotic duration was calculated in vivo by Knowlton and Widner[4] on the assumption that x ray treatment prevents cells from entering prophase but allows cells that are already in mitosis to continue until the end of telophase The number of visible mitoses counted at various intervals after treatment rapidly decreases Mitotic duration is the time taken for all mitoses to disappear The accuracy of the results would depend on how well the selected dose of x rays inhibits the initiation of prophase without affecting the evolution of mitosis The figures of these authors for mitotic duration range around 25 minutes in rat and mouse tissues From the criteria which they give for the identification of dividing cells it seems that this time refers to the period from late prophase until the end of anaphase These authors used these figures together with the mitotic index to calculate the turnover time of a variety of tissues in rat and mouse

4 Other Methods

The various methods which have been described lead to knowledge concerning the sites and rate of mitosis but provide no information as to the fate of the cells which emigrate This may be done by radioautography after injection of labeled

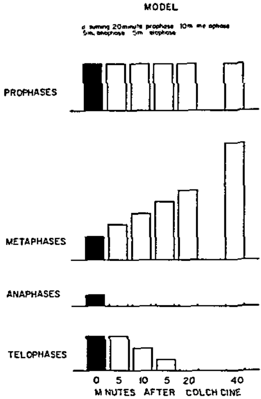


FIG 1 —Model representing the number of cells at the various stages of mitosis at successive time intervals after colchicine injection assuming a cell population with a 10 per cent mitotic index and durations of 20 10 5 and 15 minutes for prophase metaphase anaphase and telophase respectively (from Dr Stevens Hooper) Controls black columns Colchicine treated animal white columns

DNA precursors Until recently best results were obtained with adenine C^{14} (literature in reference 5) but the introduction of thymidine-H^3 in this field yields results that are more specific and give a better radioautographic localization Hence thymidine H^3 appears to be the ideal method to show areas of cellular proliferation and trace postmitotic cells[6, 7]

Other methods have been used to discover the rate at which cells leave a population or a compartment in which part of a population is held[1] For instance when radioactive iron is administered the red blood cells formed immediately afterwards contain iron labeled hemoglobin Such cells can be traced in the circulation and their turnover time in blood may be determined In fact, human erythrocytes may be said to have built in label If type O cells are transfused into a type A recipient the number surviving after various periods can be determined by counting the cells which are not precipitated by anti A serum[8, 10] The fate of labeled white blood cells transfused into a recipient animal has also been examined[11]

Conversely, the effects of removal of lymphocytes by cannulation of the thoracic duct has provided information regarding the fate of these cells Thu

Gowans[12] found that the thoracic duct in rats discharged enough lymphocytes to replace the circulating population about 11 times a day, that is, each lymphocyte spends little more than 2 hours in the blood. However, the number of lymphocytes in the collected lymph began to fall markedly after about 2 days of drainage although it could be maintained if the collected lymph, with its living lymphocytes, was reinjected intravenously. These results again place in the limelight the old theory that lymphocytes leave the blood and re-enter the lymph via tissue spaces, so that a single lymphocyte may return to the blood stream again and again.

Another approach has been to destroy the leukopoietic tissues of one parabiotic twin.[13] From the amount of blood exchanged by the 2 animals and the number of white blood cells actually found in the blood stream, the average time spent by white cells in the blood was calculated to be 170 minutes for mononuclear and 23 minutes for polynuclear leukocytes.

Hence, a variety of technics may be used to show the fate of the cells arising from proliferating populations. Only the use of mitotic counts in normal and colchicine-treated animals will be considered here.

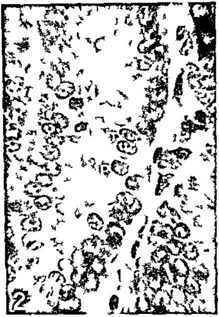
RESULTS

Interpretation of the Presence of Mitoses in Adult Animals

As pointed out above, the presence of mitoses in a cell population in adult animals reveals the existence of cell renewal, but the fate of the cells of this population may not be evident. However, in some cases, consideration of the structural arrangement makes it possible to draw conclusions or at least to make hypotheses that must be confirmed by other means. Let us take the *intestinal epithelium* of the rat as an example. This epithelium is a layer of columnar cells covering the intestinal villi and expanding without interruption down to the bases of the crypts of Lieberkuhn. The epithelium consists of two cell populations, chief cells and goblet cells, which are apparently mixed at random. Examination of this epithelium by the author in collaboration with Catherine E. Stevens[14] (who is now Stevens Hooper) showed no mitoses in the villi, since the rare dividing cells that may be observed are believed to be migrating lymphocytes. On the other hand, examination of the crypts of Lieberkuhn confirmed the well-known fact that mitoses are abundant there (fig. 2). In adult females, the weight of which had reached a plateau, the duodenum showed approximately 7 per cent of the crypt cells at some stage of mitosis.[15]

How could the significance of this high mitotic activity be established? In the past, histologists noted mitotic activity in the crypts, but were generally of the opinion that the new cells replace those cells in the villous epithelium that are damaged.[16] However, careful fixation showed us that mitoses were numerous in samples of rat intestine in which there was complete integrity of the epithelium. Hence, there must be an intense continuous cell production in the crypts of Lieberkuhn under physiologic conditions.

The fate of the excess cells was not obvious. Careful histologic study of the crypts in the rat revealed no sign of cell loss or degeneration. The lumen of the



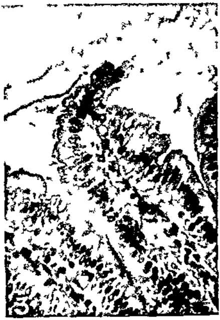


FIG 2 —Various stages of mitosis seen in a crypt of Lieberkuhn in the duodenum M metaphase T telophase P prophase (from ref 14)

FIG 3 Tip of a duodenal villus showing several cells located above the regular row of epithelial cells These cells are believed to be in the process of being extruded into the intestinal lumen [4]

crypts was narrow Indeed with the exception of the crypt bottoms it was tightly closed so that any cell or cellular debris lost to the lumen might be retained for some time and identified as such But none was found Nor were there irregularities of the epithelium The only way cells could leave the crypts would be by moving upward in an orderly fashion to become part of the epithelium covering the villi Assuming that a steady state exists the number of cells leaving the crypts should balance the number of cells added by mitosis

The next step was to examine the villi for possible clues to cellular losses No sign of loss was seen along the sides but numerous irregularities were observed at the tips of the villi Often the striated border was interrupted One or several shriveled cells were often seen Finally cells pushed above the regular epithelial lining were seen in a number of villi (fig 3) Presumably these irregularities are caused by cells which are at various stages in the process of being ejected from the epithelium Hence the name *extrusion zone* was given to them Such extrusion zones were also found in man cat rabbit and mouse and probably indicate that in all these species cells are lost from the intestinal epithelium at the villus tips

However this conclusion was no more than a histologically supported guess Evidence of cells ejected from the extrusion zone was not readily available probably because the cells in the lumen were rapidly digested by proteolytic enzymes Even so the presence of cells in the fluid collected from intestinal fistulae has been noted by several authors (quoted in reference 14) and in sections ejected cells have been seen when starved animals or isolated loops of intestine (fig 4) were examined

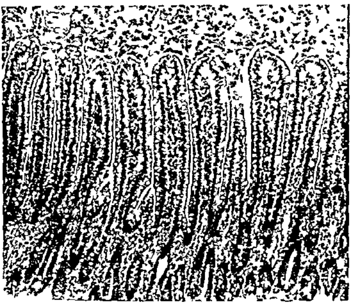


FIG. 4.—Mucosa of an isolated loop of rat ileum. The crypts are visible along the lower edge of the picture and the villi above them. Examination of the villi shows the regularity of the epithelium except for the extrusion zone visible at the villus tips. At the top the intestinal lumen is seen to be filled up with extruded epithelial cells which in places seem to be arranged in irregular columns overlying each villus tip (from Dr. Stevens Hooper).

Recently, it has been possible to label the nuclei of newly formed cells with the DNA precursors adenine C^{14} (ref. 5) and thymidine-H^3 (ref. 17). Radioautographic observations have fully confirmed the above hypotheses on the mode of intestinal renewal. This was particularly clear with thymidine H^3, since it could be shown that both chief and goblet cells arose in the crypts, ascended the villi and *fell into the lumen from the villus tips. Briefly, there is a procession of cells* from the crypts to the villus tips, presumably involving a gradual massive motion of the whole intestinal epithelium gliding lumenward to its death.

Use of Mitotic Index

In the case of the intestinal epithelium the mitotic index was calculated for the whole *epithelium (crypts and villi) and in adult female rats averaged* 3.07 per cent.[14] This information does not allow an accurate estimation of turnover time, since mitotic duration can only be guessed. If the mitosis of a crypt cell is assumed to last one hour, then 3.07 per cent of the cells divide per hour. Over a 24 hour period the number of new cells will be equivalent to 72 per cent of the number of cells initially present. Since an equal number will have been lost to *maintain a steady state, then 100 per cent of the cells will be renewed every* 33 hours (turnover time). The weakness of such reasoning is that it stands or falls on the assumption made for mitotic duration. Hence for reliable data on turnover time, the use of colchicine is required as shown below.

Information of another kind may be obtained from the mitotic index. As an *example of the possibilities let us take blood cell forming tissues. It is well* known that the cells of these tissues go through a series of generations and in so doing transform from one cell type to another. Consideration of the mitotic index of the various cell types provides information concerning the mean *life span* or generation time (i e intermitotic time + mitotic duration) of each cell type and of the *number of cell generations*

Let us examine how this was done in work on the thymus carried out by the author in collaboration with Dr Sainte-Marie[18] This work required a number of assumptions, on the basis of which the data yielded a model for lymphocyte formation in the thymus The basic assumptions are (1) Four main cell types may be accurately recognized (fig 5) the reticular cell with light cytoplasm and large nucleus up to 11 μ in diameter, the large lymphocyte with a basophilic cytoplasm and nucleus measuring more than 5 9 μ, the medium lymphocyte with a nucleus measuring 4 6 to 5 9 μ and the small lymphocyte with a nuclear diameter less than 4 6 μ[18] (2) Small lymphocytes arise from medium which come from large lymphocytes which in turn come from reticular cells (3) The life span is the same for all cells of a given type (except for some small lymphocytes which no longer divide) (4) Mitotic duration is the same for all thymus cells (5) Small lymphocytes leave the tissue in large numbers but the medium and large lymphocytes in small numbers

The problem was approached by counting resting and dividing cells of the four types

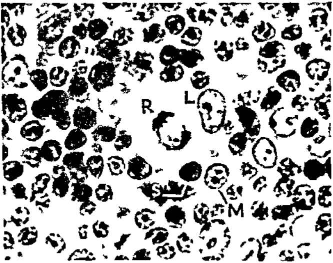


FIG 5—Cortex of rat thymus An anaphase of a reticular cell may be seen in the center (R) Left an interphase nucleus of reticular cell Right a large lymphocyte (L) Below a medium (M) and a small (S) lymphocyte (from Sainte Marie and Leblond Proc Soc Exper Biol & Med *98* 909 1958)

Let us first examine how the comparison of two cell types e g medium versus large lymphocytes makes it possible to estimate their relative life span It was noted that throughout the thymus the mitotic index of the medium lymphocytes 0 133 in peripheral cortex was twice that of large lymphocytes 0 069 in peripheral cortex Hence medium lymphocytes divide twice as often as large lymphocytes In other words the life span of large lymphocytes is twice that of medium lymphocytes (fig 6) Using similar methods it was concluded that the life span of reticular cells is eight times that of medium lymphocytes In the case of small

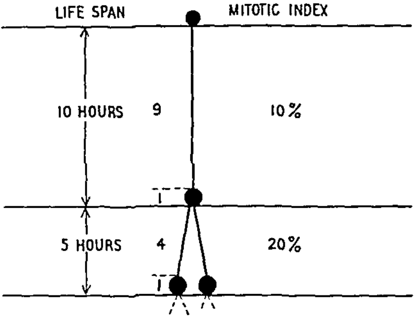


FIG 6—Model showing the relation of life span to mitotic index assuming that the mother cells have a life span of 10 hours (9 hours for interphase and 1 hour for mitosis) and the daughter cells have a life span of 5 hours (4 hours for interphase and 1 hour for mitosis) It is obvious that under these conditions the mitotic indices would be 10 per cent for the first type and 20 per cent for the second type Hence life span and mitotic index are inversely related (diagram of Dr Sainte Marie)

lymphocytes, some divide after a life span as short as that of medium lymphocytes The others live on indefinitely

The next step was *to find out how many generations of each cell type would* yield the numbers of cells and mitoses found in the counts This was done by an empirical method for the cortex of the thymus Calculations were made of how many resting and dividing cells would be expected in sections, *if one, two three* or more "medium" generations were associated with either one, two, three or more "large" generations (fig 7) It was thus found that the relative number of generations of large, medium and small lymphocytes were 4 2 2 respectively while reticular cells behaved as indicated in figure 8 (The fit between expected and experimental results is shown in fig 9, page 42)

In the thymus medulla the interpretation of the counts of resting and dividing cells was complex and had to be done in relation to histologic findings First *it appeared that the divisions of reticular cells mostly give rise to other reticular* cells instead of producing large lymphocytes, while a number of the reticular cells thus produced undergo degeneration giving rise to Hassall's corpuscles Furthermore, mitotic indices suggest that the characteristic sequence of generations observed in the cortex also exists in the medulla but the number of cells going through this sequence in the medulla is small [19] On the other hand, a number of *medium and an even larger number of small lymphocytes migrate from the cortex* to the medulla and from the medulla into the lymphatic and blood vessels, so that the medulla serves as the pathway for thymic lymphocytes to reach the circulation (*fig* 10 *page* 43)

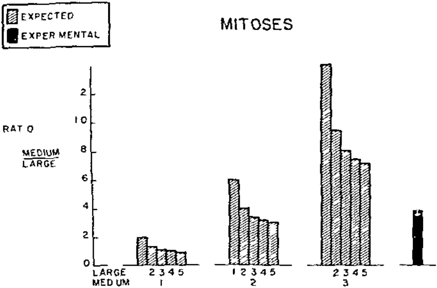


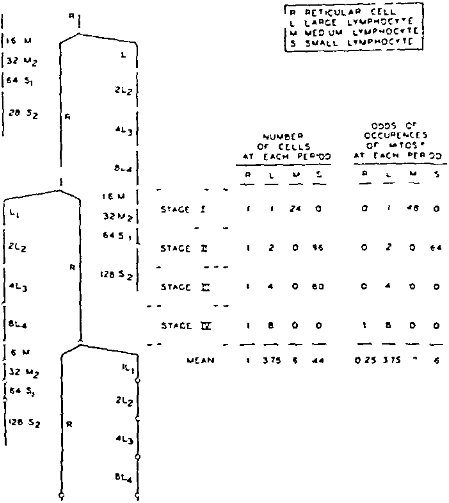
Fig 7 Diagram showing the ratio of the number of mitoses in medium to that in large lymphocytes The experimentally obtained value (black column at right) is compared to the values to be expected for 1 2 or 3 generations of medium lymphocytes combined with 1 5 generations of large lymphocytes (gray columns) It was concluded from the comparison that 2 generations of medium were combined with 3 or 4 generations of large lymphocytes (although the possibility of 2 or 5 generations of large lymphocytes could not be excluded by consideration of these data alone diagram of Dr Sainte Marie)

In conclusion it is possible to obtain information on the life span and number of generations of cells from a consideration of counts of resting and dividing cell This information coupled with histologic investigations revealed the mode of cell production in thymus Nevertheless the figures for the life span of the various cell types were relative to one another Absolute figures may be obtained by a different technic now to be described

Use of Colchicine

Colchicine may be used to estimate not only mitotic duration but also turnover time The turnover time of a cell population is usually equal to the mean life span of the cells making up that population For simplicity s sake let us assume that, starting at the time of injection colchicine stops all metaphases from transforming into anaphases so that the number of colchicine-blocked metaphases is equal to the number of cells entering mitosis between injection and sacrifice If mitosis has a normal frequency of p per cent, and colchicine blocked metaphases have a frequency of q per cent, t hours after injection the mitotic duration m is pt/q hours and the turnover time (time for 100 per cent renewal) is 100 m/p hours If m is replaced by pt/q in this formula the turnover time becomes 100 t/q hours thus the turnover time may be estimated if only the count in colchicine-treated animals q is known The use of such methods in the case of the intestinal epithelium yielded a turnover time of 37 7 hours for the duodenum that is 1 57 days[14]

The use of colchicine is fraught with difficulties which were not always recog



	NUMBER OF CELLS AT EACH PERIOD				ODDS OF OCCURENCES OF MITOSIS AT EACH PERIOD			
	R	L	M	S	R	L	M	S
STAGE I	1	1	24	0	0	1	46	0
STAGE II	1	2	0	96	0	2	0	64
STAGE III	1	4	0	80	0	4	0	0
STAGE IV	1	8	0	0	1	8	0	0
MEAN	1	3.75	6	44	0.25	3.75	[illegible]	6

Fig. 8.—Stem cell renewal theory of lymphocyte formation in thymus. Diagram representing the most probable pattern for the filiation of thymic cells in the cortex. Each reticular cell at regular intervals yields large lymphocytes that pass through 4 generations and then produce medium lymphocytes that undergo 2 generations, thus giving rise to small lymphocytes of which there are also 2 generations (the vertical dimension is time). This diagram was prepared from data obtained in the peripheral region of the cortex. The numbers of cells and the odds of occurrence of mitosis at each one of the stages are obtained by inspection of the corresponding portion of the diagram. The means (lower right) are used to calculate the "expected" ratios reported in figure 9 (from Sainte-Marie and Leblond, Proc. Soc. Exper. Biol. & Med. 97: 263, 1958).

mized by earlier investigators, including the writer. As a result, a somewhat pessimistic attitude regarding the significance of colchicine results has often been adopted.[1] Adequate results, however, may be obtained with colchicine, if series of treated animals are sacrificed at several time intervals after injection. In this manner, it is possible (1) to determine whether the effect of colchicine on the organ investigated follows the theoretical model in figure 1 and, if so, (2) to fit a straight line to the increasing metaphase counts and thus estimate at what rate metaphases accumulate. From this rate, it is easy to calculate mitotic duration and turnover time.

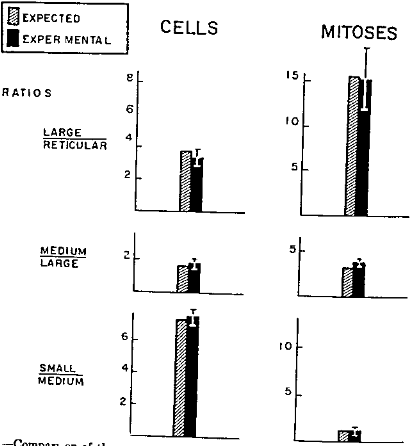


FIG 9—Comparison of the experimental ratios of numbers of cells and mitoses (black columns) with the ratios expected on the basis of the diagram in figure 8 (gray columns) The agreement is good

In an experiment of Stevens Hooper male rats weighing about 260 Gm were killed 1 1 5 2 2 5 and 3 hours after injection[3] The percentages of prophases metaphases anaphases and telophases are shown in figure 11 The data conform closely to the model shown in figure 1 A straight line fitted to the metaphase counts shows that metaphases accumulate at a rate of about 3 38 per cent per hour if a correction is made for the delay of roughly $^1{}_2$ hour required for subcutaneously injected colchicine to take effect this figure becomes 4 13 per cent per hour This result gives a turnover time of 24 2 hours for the cells of the crypts alone and about 2 days for the renewal of the whole epithelium which result agrees well with recent estimates made with H^3 thymidine

The duration of individual mitotic stages with a normal frequency of p per cent while colchicine blocked metaphases have a frequency of q per cent t minutes after injection may be calculated from the formula pt/q used to figure mitotic duration This gives the following values prophase 1 to 1½ hours metaphase 19 2 to 21 75 minutes telophase 43 to 53 minutes that is over 2 hours for mitosis to be completed

Some of the complications that may arise with colchicine were shown by O Héroux[20] who used it to study mitotic rates in the epidermis of rats under warm

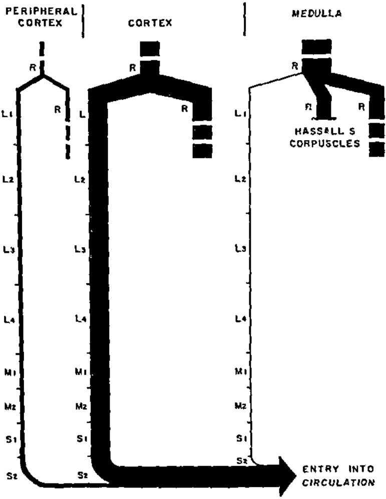


FIG. 10.—Formation and migration of lymphocytes in peripheral and deep cortex and in medulla of rat thymus (from ref. 19). At the left, the complex pattern represented in figure 8 and derived from the peripheral cortex data is presented in simplified form, indicating that a reticular cell (R) divides to yield on the average one large lymphocyte (L_1) and another reticular cells (R). The basic pattern is repeated in deep cortex and medulla, *with the thickness of the long vertical branches made proportional to the rate of local* lymphocyte production. In medulla, few large lymphocytes are produced from the mitoses of reticular cell, most of which yield new reticular cells. On the average, half of these degenerate and die, usually as Hassall's corpuscles. At the base of the diagram, the length of the horizontal portions in the 3 zones were made proportional to the mean time spent by the mature, nondividing small lymphocytes (S_2) in each zone. The portion corresponding *to peripheral cortex joins the line standing for deep cortex, and both together join the line* standing for medulla, thus indicating the pathway of lymphocyte migration from peripheral to deep cortex and then to medulla, from which the cells reach the circulation.

and cold conditions. He found that 0.1 mg. colchicine per Gm. body weight blocked metaphase effectively in all parts of the skin in rats kept at 30° C. However, at 6° C., the same dose was effective only in such tissues as back skin and duodenal epithelium, which were not chilled. In the ear and foot epidermis, which were many degrees below central body temperature, no substantial increase in the number of metaphases was observed until this dose had been

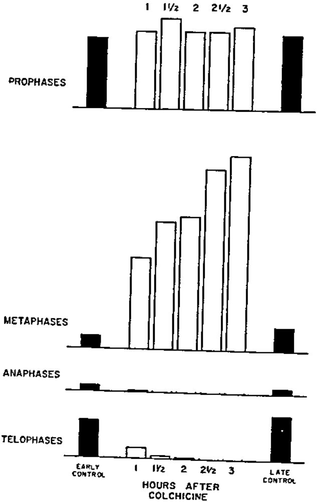


FIG 11 —Data of Stevens Hooper on the frequency of mitotic division in the ileum of the white rat as shown in controls (black columns) and at various time intervals after colchicine injection (white columns) The data show no significant change in the frequency of prophases a progressive increase in the number of metaphases and a decrease in the number of anaphases and telophases with time

repeated 4 times in 24 hours, and even then telophases had not completely disappeared (fig 12)

As a fivefold increase in dose did not increase the rate of accumulation of metaphases or disappearance of telophases, it seems unlikely that this is a result of the escape of metaphases from the blocking effect of colchicine The other explanation is that cells enter mitosis at such a low rate in the cold that mitotic duration greatly exceeds the time during which colchicine is effective Using his data on tissues in which colchicine produced a significant increase in the metaphase count Héroux calculated the rate at which cells enter mitosis—the induction rate—and the duration of mitosis He then expressed these values as functions of tissue temperature, by fitting straight lines on a semi log plot The lines so obtained showed that mitotic duration has a Q_{10} (change with each 10° C) of

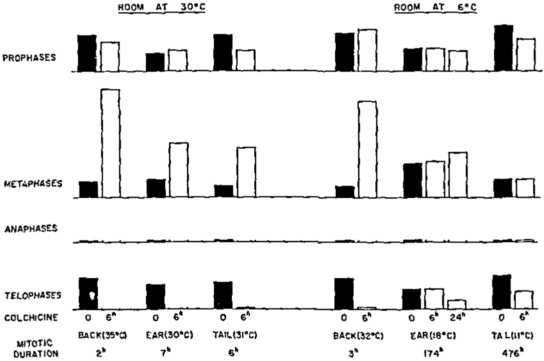


FIG. 12.—Data of Héroux on the frequency of mitotic division in various regions of the epidermis of the rat at a room temperature of 30°C (left hand side) and 6°C (right hand side). At the base the regions from which the epidermis was investigated are listed with the temperature of the tissue in brackets. The last line reports the mitotic duration calculated from the colchicine data. It is apparent that at 30°C, the frequency of metaphases was less in the more exposed and therefore colder epidermis of ear and tail than in the warmer epidermis of the back. Figures for mitotic duration are correspondingly higher in ear and tail epidermis than in back epidermis. These phenomena were greatly emphasized at 6°C. *The estimated mitotic durations in the ear and tail epidermis were 174 and 476 hours respectively.*

10. *The relation obtained in this way was used to calculate mitotic duration and induction rate for the two cases, ear and foot epidermis at 6° C., for which they* could not be calculated directly because of the failure of colchicine to produce a significant increase in the number of metaphases. The results for mitotic duration are at the bottom of figure 12.

Recently, the existence of diurnal variations in mitotic activity have been emphasized[21, 23]. It is therefore important to estimate an average mitotic rate in order to have an accurate estimate of turnover time. The method proposed for the epidermis[24] and lung[25] was to use 4 groups of animals sacrificed at various times over a 24 hour period, but always 6 hours after colchicine injection. For instance, in the study of the epidermis, the 4 groups were injected at 4 A.M., 10 A.M., 4 P.M., and 10 P.M. All animals were sacrificed exactly 6 hours after injection. The sum of the mean number of mitoses in these 4 groups supplied the mean number of mitoses per day. Thus, in table 1, derived from measurements carried out on the plantar epidermis in 250 Gm. rats kept at 27° C., the number of mitoses per day was 5.24 per cent of the total number of cells present in the Malpighian layers (stratum spinosum and granulosum). Hence, the turnover time

TABLE 1 —*Per Cent Metaphase in Malpighian Layers of Epidermis 6 Hours After Colchicine in the Rat*

Time of colchicine injection	Time of sacrifice	Per cent metaphases
4 AM	10 AM	1 63
10 AM	4 PM	1 48
4 PM	10 PM	1 22
10 PM	4 AM	0 91
Total		5 24*

* = % cells dividing in one whole day
Turnover time = 19 days

of the cells in this region is 19 days Improved results might be obtained by applying the method of Stevens Hooper[3] at 4 or 6 different time intervals during a 24 hour period

CONCLUSION AND SUMMARY

The sites of cell proliferation in the adult animal may be investigated by means of mitotic counts with or without colchicine treatment

A rough assessment of the rate of cell production is provided by the *mitotic index* With some tissues, such as the thymus the mitotic index may be used to calculate the relative life span of the cells and the number of generations in the population

A precise determination of the rate of cell production may be obtained by means of a somewhat refined *colchicine technic* which yields mitotic duration as well as turnover time of the cell population An average figure for the turnover time over a 24 hour period may be obtained by using animals sacrificed at 4 or 6 time intervals during the day

While mitotic counts with or without colchicine treatment do not make it possible to trace the migration of the cells that have undergone division as may be done by radioautography after administration of DNA precursors such as tritium labeled thymidine it is felt that at the present time the colchicine technic is best for estimation of mitotic duration and turnover time

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Discussion

E P CRONKITE Dr Leblond, we have observed an outpouring of potentially dividing cells from the lymph nodes which represents a continuous loss of such cells from the node unless there is a constant recycling If there is comparable loss from the thymus, how would this influence your computations?

C P LEBLOND Before answering your question I would like to emphasize two points which may have confused the audience First, it is important to realize that what various authors call a small lymphocyte may not be the same thing Dr Sainte Marie and I decided to consider that, in sections, cells with nuclei having a diameter less than 4 6 μ are small lymphocytes We then found that there are two generations of cells smaller than this limit so that if our definition is accepted there are two generations of small lymphocytes We see prophases metaphases anaphases and telophases that are small enough to be attributed to these cell In any case any one who speaks of small lymphocytes should provide an operational definition or description of the cells he has in mind Secondly there may be a recirculation of lymphocytes but lymphocytes which have left the lymphatic organs do not seem to return to the thymus in significant numbers The thymus has few blood vessels The abundance of mitoses is such that the population pressure tends to push cells out rather than letting them in

Finally to answer your question the model which we published[1] is based on the assumption that one cell at least leaves the thymus the small lymphocyte (S) Hence our calculations imply a cell loss Does such a statement answer your question?

E P Cronkite No it really doesn t Cells capable of division are pouring out of lymph nodes They are proliferating cells as measured by in vivo and in vitro labeling with tritiated thymidine This represents a loss in the proliferative compartment Now unless cells of the same type return in equal numbers to the tissue it would appear to me that this would drastically influence your type of analysis based on ratios of cell types and mitotic index

C P Leblond While the number of small lymphocytes that leave the thymus is taken into account in the model[2] there are also medium and even large lymphocytes leaving The number of those migrating out may be high but not enough to influence our calculations (cf [2])

Incidentally examination of the few large and medium lymphocytes found in the lumen of lymphatic channels indicates that these cells continue dividing just as they do in the thymus proper In other words the escaped large and medium lymphocytes continue yielding small lymphocytes

The lymph nodes are a more complex problem In view of the pyknosis seen in some germinal centers in lymph nodes it is possible that some of the reticular cells and large lymphocytes that divide there (as Dr Yoffey showed in his striking pictures of labeled cell) may die very soon after they have appeared just as the reticular cells do in the medulla of the thymus

J M Yoffey We have been intrigued by the statements that germinal centers are reaction centers with extensive pyknosis Our experience has been that in a good healthy colony we see singularly few of these dying cells Frankly we don t believe that you find very many of these cell dying in healthy animals There is no doubt however that they are quite readily damaged and that pyknotic changes then become very evident Whatever may happen to the cell in the gland we obviously can t deny that some of them may die The escape of these labeled cells into the lymph is a different proposition In the lymph they certainly don t look like dying cells We are endeavoring to prove or disprove that in the lymphoid tissues there is a special type of cellular development a series of rapid divisions which I call inactivation By this mechanism your primitive cells are reduced to a relatively small and inactive state the small lymphocyte for mobilization and transport through the blood stream Then in the appropriate environment and with the appropriate stimulus you get the reverse change a reactivation with rapid enlargement and the primitive stem cell emerging once more

W Dameshek There is a marked qualitative and quantitative difference between an erythroid cell and a reticulo-epithelial cell Does Dr Yoffey think that these changes go on from moment to moment as it were in a dynamic tissue like lymphoid tissue

J M Yoffey It certainly is a dynamic sequence of events You start off with a primitive cell I am not calling it reticular The term reticular has been fraught with such difficulties that I avoid it But you start off with a primitive cell which goes through a number of divisions on the lines that Drs Leblond and St Marie have demonstrated so nicely in the case of the thymus[1, 2] I don t know whether we have eight generations in the lymph node but there is a continuous dynamic process and the cells at this stage are genetically very plastic It is at this stage where the cell if it gets the appropriate stimulus can go on to a specialized type such as a plasma cell but the normal line of development in the absence of such a specific stimulus is in the direction of forming these small lymphocytes You can see all intermediate stages It is the existence of the precursor cell pool in a dynamic state which makes it very difficult to do precise quantitative work with thymidine If you give a single dose of thymidine, you can label different cells of the pool at all stages, but the most primitive cell will take eight days before its descendants (in eight generations, 256 cells) would finally appear in the thoracic duct lymph as small lymphocytes

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The Quantitative Estimation of Mitotic Growth in the Bone Marrow of the Rat by the Stathmokinetic (Colchicinic) Method

By PIERRE DUSTIN JR

THE SEPARATION of mitotic chromosomes under the influence of the spindle may be selectively disturbed by a group of chemicals, the spindle poisons The most widely known are colchicine and its derivatives podophyllotoxin and peltatin and various arsenical compounds such as arsenious oxide and sodium cacodylate They totally or partially destroy the fibrillar structure of the spindle, *the resulting atrophic or amorphous spindle (pseudospindle) is unable to* move the chromosomes which lay in a state of pseudometaphase or stathmokinesis * New mitoses however continue to appear for some hours at the same rate Arrested metaphases accumulate and the mitotic index is considerably increased

The first authors[1] [12] who studied this effect considered that the mitotic index i_t^* found in a tissue t hours after the action of a stathmokinetic drug such as colchicine was directly related to t and to the mitotic index i of controls and inversely to m the unknown duration of mitosis *before* the action of the drug From this relation $i_t^* = (i \times t/m)$ the duration of mitosis is easily calculated for $m = (i \times t/i_t^*)$ However this is only possible if several conditions are met *a circumstance which is exceptional in the complex tissues of mammals*[8]

In the present paper we will analyze some results on the growth of bone marrow cell The possibilities and the limitations of the stathmokinetic method will be made clear and the discussion of our results will provide an opportunity to consider other papers in which the duration of mitosis has been calculated in spindle poisoned cells

MATERIAL AND METHODS

Stock albino adult rats were used throughout As a mitotic poison desacetyl *N* methyl colchicine (colcemid or demecolcin Ciba) was injected intraperitoneally in doses of 1 mg /Kg Bone marrow smears were fixed in absolute alcohol *and stained by the method devised by de Harven*[3] *Eosinophilic mitoses* were counted separately The granulocytic and the erythroblastic mitoses were counted together since there was no indication of important differences between their mitotic rates The variations of mitotic index in the bone marrow were compared to those observed in the thymus the spleen and the small intestine after routine sectioning and staining All animals were killed by ether One group of rats was adrenalectomized and kept on a normal diet for two days with a 1 per cent solution of sodium chloride as drinking water In the interval of two days adrenal regeneration did not take place since great care had been taken to remove all the periadrenal fat

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* From the Greek *o stathmos* the station the arrest

RESULTS

The results, based on the figures published by Piscitelli et al.,[15] are summarized in figure 1. For each time interval after the injection, and in noninjected controls, the average mitotic counts were made for groups of four or five animals.

If we first consider the relative *percentages* of prophases, metaphases, anaphases, and telophases, *we see that during the first 30 minutes after injection, most of the existing ana-telophases are completed.* The percentage of prophases does not change during this period, but after one hour it decreases considerably. This is a simple consequence of the accumulation of arrested metaphases. Piscitelli[15] has shown that during the experiment, the number of new cells entering prophase is not appreciably changed.

After six hours, some ana-telophases reappear. This is proof that the duration of complete spindle poisoning does not exceed this period. Hence, any quantitative *study of growth in bone marrow with colcemid should be done within less than six hours from the beginning of the experiment.* At eight hours, it is clear that there is no more spindle poisoning, the mitotic phase percentages being the same as before treatment.

If we now consider the *mitotic index*, expressed here as the number of mitoses (normal or arrested) per 1000 cells, we see that during the first half hour after injection, this index drops notably. After one hour it is about the same as at the *start of the experiment. This transient decrease reflects the disappearance of the* telophasic cells, which end their mitosis. The metaphase index (number of metaphases per 1000 cells) is not modified during the first half hour, the increase in the *percentage* of metaphases compensating for the fall in the total number of

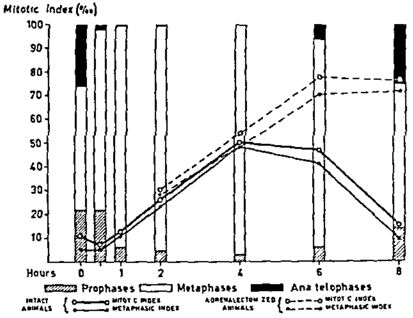

FIG. 1.—Variations of the mitotic index and the percentage of mitotic stages in the bone marrow of normal and adrenalectomized rats (from Piscitelli, de Harven, and Dustin[14]). The mitoses of eosinophil granulocytes are not included.

mitoses Soon however the number of mitoses and metaphases begins to rise and it is clear that this increase is roughly linear during the first four hours Hence any quantitative analysis of cellular growth should consider mainly the mitotic and metaphasic index curves between one and four hours After four hours at the time when the mitotic arrest ceases to be effective the number of mitoses decreases rapidly and after eight hours is almost normal

The dotted lines (fig 1) indicate the behavior of adrenalectomized animals injected with the same dose of colcemid The slope of the mitotic index curve is the same as in the injected intact animals but the action of colcemid is much longer At six hours the number of arrested metaphases still rises Beyond a six hour period considerable variations are found from one rat to another and in fact the spindle poisoning lasts for more than eight hours in certain cases It has been concluded that the adrenal hormones help the cells to overcome the spindle inhibition caused by drugs such as colcemid and other spindle poisons [5] Adrenalectomized rats when injected with cortisone behave like intact rats The favorable effect of cortisone on the spindle function is comparable to its action on muscle contraction [3] This effect has been also demonstrated in other tissues of the rat and in particular with the hair bulbs [4]

From this data how can we calculate the duration of mitosis? It seems preferable to consider the period extending from one to four hours for the following reasons (1) the mitotic arrest *is complete during that entire period—no more* ana telophases are visible (2) the linear increase of the mitotic index suggests a simple accumulation of mitotic figures In three hours the average mitotic index rises from 12 per thousand to 50 per thousand Supposing that the total number of bone marrow cells has not changed during this short time the mitotic duration calculated from the formula $m = (i \times t/i')$ would be equal to $(12 \times 3/50) = 0.72$ hours $= 43$ minutes

The above figure can be checked by calculating from the metaphase index instead of from the mitotic index The former rises during the same period from 5 to 45 A similar calculation shows that the metaphase lasts for 20 minutes. The metaphasic percentage in controls is about 50 per cent The percentage of any given stage being proportional to its duration the entire mitotic cycle should last 40 minutes which is in agreement with the duration calculated above

If the mitotic index is directly proportional to the duration of mitosis and about 1 per cent of all cells are undergoing mitosis at any moment the length of intermitosis should be one hundred times longer than that of mitosis i e 72 hours This means that the duration of the experiment is considerably shorter than the mitotic cycle

These experiments also provide data about the relative mitotic growth of eosinophilic leukocytes and other cells The increase of the mitotic index of eosinophils is one third of that of the other bone marrow cells and the calculated duration of the eosinophil mitoses was found to be about 120 minutes [5] On the other hand the mitotic index of the eosinophils in noninjected animals is about the same as that of the other cell If their mitosis lasts three times longer their mitotic index should be three times higher unless one supposes that the intermitotic time for eosinophils is also three times longer This however can only be suggested *with caution for it entails the comparison of a complex population of cells* of one type—the eosinophils—and of a still more complex population of cells of two principal types—neutrophil granulocytes and erythroblasts. Nevertheless, as no evidence of "resistance" of the eosinophils to the action of spindle poisons has been observed, the very slow rise of their mitotic index after spindle poisoning can only be explained by the longer duration of their mitosis.

DISCUSSION

The first authors who studied the mitotic increase in tissues after the injection of colchicine[1, 12] believed that there was a simple relationship between the number of mitoses found after the action of the drug and the duration of mitosis before this action. This opinion is correct only in certain theoretical conditions.[8] Does a complex tissue such as rat bone marrow permit a study of this type? The first condition is that the stathmokinetic poison should arrest all mitoses rapidly after its injection into the animal. Figure 1 shows that all mitoses are effectively brought to a standstill within one half hour. Because the increase of the number of metaphases is linear from 30 minutes to 4 hours after injection, which indicates a gradual accumulation of abnormal metaphases, we can base our calculations upon these figures.

The second condition is that the intermitotic period should be very much longer than the duration of mitotic arrest. From the calculated figure of 72 hours, it would seem that this condition is fulfilled. However, some cells may have a much shorter intermitosis but it is unlikely that it should be as short as four hours. This is also well demonstrated by the results obtained in adrenalectomized animals. The progressive decrease of the stathmokinetic index after four hours is not the result of a short intermitotic period, nor is it due to the absence of cells capable of entering mitosis, as Journoud[11] proposed. To the contrary, after adrenalectomy the mitotic count rises at the same rate for at least six hours, and longer in some animals.

This action of the adrenals brings into the foreground another condition: the drug must not destroy any cells. It is known that most spindle poisons—and colcemid is no exception—do cause cellular destruction after the metaphase arrest. Piscitelli[15] has shown that in the experiments related above such a destruction takes place. After six hours, there is no increase in the number of anaphases and telophases, as would be expected if the arrested mitoses were to complete their cycle once the spindle recovered. Most of the poisoned metaphases appear to be destroyed. This is important in understanding the decrease of the mitotic index after four hours but does not appear to modify the gradual increase of the stathmokinetic (metaphasic) index during the first four hours. Actually, cellular destruction can easily be overlooked in a complex tissue like the bone marrow, especially if it is rapid.

Another cause of error is that the bone marrow cannot be considered homogeneous from a mitotic point of view. It is impossible to know whether all the cell divisions proceed at the same speed. It has already been pointed out that one cell type, the eosinophil granulocyte, has mitoses which apparently last much longer than those of other cells. Also, in a differentiating tissue it is most probable that the duration of the intermitotic period varies according to the stage of differentiation.

Lastly, while it can be assumed that the bone marrow is in a stable state i e , the number of cells produced by mitotic growth equals the number of cells leaving the bone marrow this is not certain after the action of colchicine This has long been known[6] to rapidly increase the number of leukocytes in the circulating blood by a mechanism which remains unexplained Considerable errors could appear in our calculations if colchicine sweeps a great number of cells out of *the marrow as some authors believe it does*

Consequently even when the arrest of mitoses by colchicine or any similar poison is complete and the increase of the mitotic index is linear the precise estimation of mitotic duration is only theoretically possible

Another difficulty may arise from the diurnal changes of mitotic activity This has not been described in bone marrow but is important in other tissues Muhlemann et al[14] have demonstrated the influence of the diurnal rhythm on the mitotic arrest by colcemid in rats They compared the effect of the mitotic poison nine hours after injection in day (resting) animals and night (active) animals Whereas the increase of metaphases in the duodenal crypts was about the same in the two groups (fivefold in day animals sixfold in night animals) the differences were considerable in thyroid and buccal epithelium In the thyroid there was no apparent change in the mitotic index in day animal while it increased sixfold in night animals In the mouth epithelium the difference was still greater In day animals the mitotic index rose from 45 7 to 85 6 In night rats the rise was from 8 9 to 107 5

No conclusions about the duration of mitosis can be derived from these figures In the day animals while the number of mitoses increased under the effect of colcemid the number of new cells entering prophase must have decreased considerably *In the night animals the abnormal increase after mitotic poisoning* can be explained by two factors the physiologic stimulation of mitosis and the accumulation of arrested metaphases If such figures were utilized to calculate the duration of mitoses for buccal epithelium in the day group the duration of mitoses would be about 4 hours and in the night group about 40 minutes In any tissue which shows diurnal variations stathmokinetic poisons cannot be used for such purposes On the other hand it is evident from these results that colcemid provides an interesting *tool* for demonstrating the importance of the diurnal changes

Similar difficulties arise when colchicine is used in hormone-stimulated tissues[8] Many discussions about the mitotic stimulating properties of colchicine could have been avoided if the changes of the mitotic rhythm in the control animals had been more carefully considered The colchicine method of measuring the duration of mitosis in a tissue where mitotic index is increasing leads to results which contradict what is known about the duration of mitosis by other technics This applies for instance to the first experiments of Allen et al[1] who calculated mitotic durations of less than 15 minutes to those of Leblond and Allen on pigeon crop glands stimulated by prolactin (16 min) and to the last paper published by A P Dustin[7] who also found a considerably increased mitotic index This implied either that colchicine stimulated mitosis or that the duration of mitosis was less than 15 minutes In all these experiments colchicine acted at the same time as a powerful hormonal stimulant of cell division Other methods for measuring the duration of mitosis and intermitosis with spindle poisons have been proposed such as the rate of *disappearance* of normal mitoses in tissue cultures treated by colchicine[2] It is known that under such conditions the spindle effect is never immediate beginning only 30 minutes after the start of the experiment The disappearance of the *normal mitoses follows an exponential law It is easy to measure that period of time after which half of all normal mitoses have* ended The authors compare this half time of metaphases with those observed with radioactive elements Such a comparison is open to criticism for the duration of mitosis varies within close limits and cannot be compared to the decay of radioactive atoms which varies between 0 and infinity The calculated values agree however rather well with those of other authors The duration of metaphases in megaloblasts is found to be 10 minutes The percentage of metaphases being 20 per cent the duration of the whole mitotic cycle would be 50 minutes *The objection to this method is that it does not depend on positive facts but* upon negative ones i e the *absence* of normal mitotic figures This means that the method can be used only under certain conditions as with cultures in vitro where by the action of colchicine the spindle vanishes completely In injected animals arrested metaphases may have the so-called star appearance which indicates the persistance of a small but inactive spindle The distinction between star metaphase and normal ones can be quite difficult To this cause of error *we must add the difficulty of determining the length of the period of latency,* which *may* not always be so clear cut as in the example given by *Astaldi* and Mauri[2]

Other aspects of the stathmokinetic method are not discussed here and in particular the case of exponential growth which is often encountered in rapidly growing tissues tumors plant meristems etc A mathematical treatment of the significance of the mitotic index in such conditions has been given by Hoffman[10] *For this author the mitotic index is no longer the relationship between the duration of mitosis and intermitosis but rather this relationship must* be divided in the simplest conditions by ln2 = 0 693 For instance in experiments where Lettré[3] estimated the mitoses to last 15 minutes with the colchicine method the correct figure would be 24 minutes

Mention should be made of the careful mathematical analysis by Evans et al[9] of the growth in root meristems A contradiction can be found in their results whereas the calculated value for the duration of *mitosis is* 3 9 ± 0 2 hrs *the mitotic index nearly doubles between the first and the fifth hour after the* action of colchicine This would mean in our opinion that the average duration of mitosis is about two hours These authors believe that the accumulation of mitoses after colchicine is an exponentially increasing function of time However their graph for three different concentrations of colchicine acting during four hours shows a linear increase of metaphases

SUMMARY

The mitotic arrest by desacetyl N methyl colchicine in the bone marrow of the rat is given as a model to be used for calculating the mitotic duration by the stathmokinetic method The limitations and difficulties of this have been discussed The complexity of measuring mitotic durations in tissues is underlined

Even if stathmokinetic poisons are unreliable from a quantitative point of view, they remain an excellent tool for the study of mitotic growth The results obtained, however, should always be checked by other technics

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Discussion

A S GORDON Dr Dustin, one of your slides indicated a persistence of the marrow metaphase stages in adrenalectomized rats treated with colchicine Our laboratory as well as that of Crafts, has demonstrated the development of an anemia in salt-maintained, adrenalectomized rats, associated with a reduction in the absolute numbers of nucleated red blood cells within the marrow. It seems possible that your observations may shed light on the mechanisms underlying the anemia and erythroid marrow hypoplasia in adrenalectomized animals. I would like to ask you how adrenal cortical steroids affect the mitotic block produced by colchicine.

P. DUSTIN: In an adrenalectomized animal, mitoses are normal. However, when colchicine is given, the duration of mitotic arrest is much longer. If you give these animals cortisone, then, the duration of mitotic arrest returns to normal. It appears as if cortisone is in some manner useful for the spindle function. This may be related to the observations which show that cortisone is also useful for muscular contraction. Spindle function has often been compared to muscular function.

Determination of Leukocyte Pool Size from Parabiosis and Cross-Transfusion

By D C Van Dyke and H G Parker

FROM THE fact that cross transfusion of leukocytes in large numbers from a normal to an aleukopoietic animal fails to equalize the leukocyte count of the two animals, *we know that either leukocytes have a very short survival time* in the circulating pool, or that they are being diluted in an exceedingly large pool that is in equilibrium with the circulation or a combination of the two How much more than this can be learned from the cross transfusion experiments?

The parabiotic cross transfusion experiment that we reported several years ago[3] involved a relatively slow rate of exchange of blood from a normal rat to an aleukopoietic (10 times the blood volume per day) and an observation period of sufficient duration It was assumed that on the fourth day of the experiment a steady state had been reached In a steady state, the ratio of the leukocyte level of the producing to the nonproducing partner is a function of (1) the rate of transfer of blood between the pairs, (2) the volume in which the leukocytes are diluted and yet remain in equilibrium with the circulation or the leukocyte 'space "[*] and (3) the average time the leukocyte spends in the leukocyte space Although we know the leukocyte levels and the rate of exchange of blood between the partners, we need to know the leukocyte space in order to calculate the average time a leukocyte spends in equilibrium with the circulating blood, or we need the average life to calculate the size of the leukocyte space However, by knowing the leukocyte count and the rate of exchange of blood, a simple calculation shows that the nonproducing partner had a net gain of 12×10^6 *leukocytes per hour to maintain* 14×10^6 leukocytes *present in the circulating* blood at any one time, or a total time spent in the circulating blood of approximately *one hour* I wish to re emphasize that from these data alone one cannot determine to what extent the leukocytes are in equilibrium with extra circulatory pools or with large numbers of leukocytes sequestered within the vascular bed The average time of one hour represents *the total intracirculatory* time, possibly including several migrations in and out of the circulating pool If the leukocyte space were 10 times the blood volume, the average time spent in the total leukocyte space would be 10 hours rather than 1 hour From these data alone one cannot determine the total number of leukocytes in equilibrium with the circulating pool

Pool size can be determined by observing the effect of adding a diluent, by observing the dilution of added material, or by observing the effect on concentration of adding or removing a known number of cells Hollingsworth and Finch[1, 2] have done a large amount of very careful work in which they have varied the

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This work was supported by the U S Atomic Energy Commission

[*] To be defined in the same way as is sodium space i e the theoretical volume in which the leukocytes would be distributed if the concentration were equal to that of the circulating blood throughout

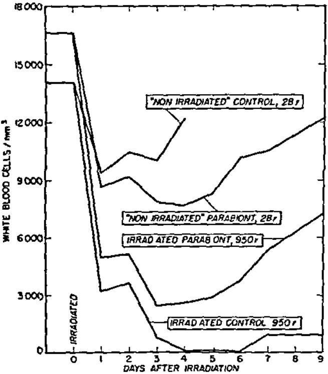

FIG 1 —White cell levels of the blood of parabionts and controls following irradiation (*from Van Dyke and Huff*) [2]

experimental model in a number of ways so as to give some idea of the size of the leukocyte space by the effect of dilution on the leukocyte concentration. By rapid exchange transfusion with leukocyte free blood containing labeled erythrocytes it was demonstrated that the animal's erythrocyte dilution rate was 3.5 times as rapid as the rate of fall of the *leukocyte concentration, indicating that the immediately exchangeable leukocyte pool is 3.5 times* as large as the red cell pool. This may be mobilization from the capillary walls, from the tissue spaces,

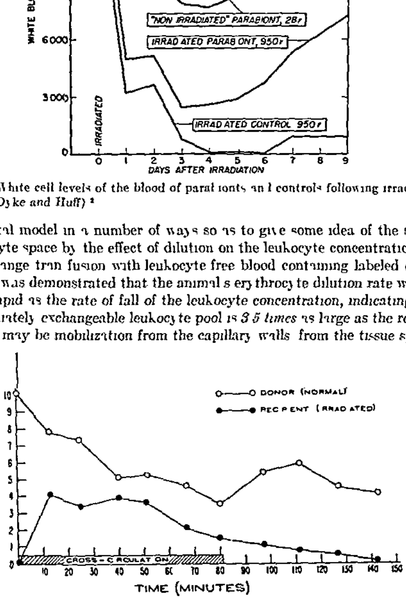

FIG 2 —Arterial blood leukocyte counts during and after a typical cross circulation experiment (from Hollingsworth, Finch and Chang) [3]

or from any other contributor to the immediately available leukocyte pool, but the total number of leukocytes contributing to this pool must be 3 5 times those actually present in the circulating blood This experiment was done with an extremely rapid rate of exchange (one blood volume per minute), the entire experiment taking only an average of four minutes, so that the influx of leukocytes from ultimately exchangeable pools would be negligible

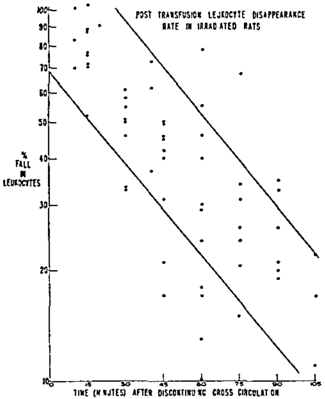

FIG 3 —Fall of arterial blood leukocyte counts of irradiated rats after cessation of cross-circulation (from Hollingsworth Finch and Chang) [2]

These authors did a series of cross transfusions comparable to the previously mentioned parabiotic experiments, except that with the aid of a mechanical pump the rate of exchange of blood was 24 times as rapid as that in surgical parabiosis (10 times the blood volume per hour) If one takes the number of leukocytes transferred to the aleukopoietic partner, minus the number returned, one obtains a net loss of 420 × 10^6 leukocytes

The average concentration in the donor was 6 × 10^6/ml and in the recipient was 3 × 10^6/ml The average weight of the pairs was given as 250 to 400 which means a blood volume of 15 ml and the rate of transfer of blood was 180 ml in the 80 minute period of the experiment Thus 840 × 10^6 leukocytes entered the aleukopoietic animal during the 80 minute period and 420 × 10^6 were returned giving a net loss of 420 × 10^6 leukocytes during the 80 minutes of observation

The loss of this number of leukocytes reduced the concentration in the donor by 65 per cent. Thus, 420 × 10^6 leukocytes is 65 per cent of the total present, giving 640 × 10^6 leukocytes present at the beginning of the experiment. This is 4.3 times as many leukocytes as were present in the circulating blood at the beginning of the experiment, indicating that the available leukocyte pool is effectively *4.3 times* the blood volume. The difference in the previously obtained value of 3.5 and this value of 4.3 can be explained by the fact that during the 80 minutes required for the experiment, a certain number of leukocytes entered the immediately exchangeable pool from the ultimately exchangeable leukocyte pool. The rate of exchange between them will be considered later.

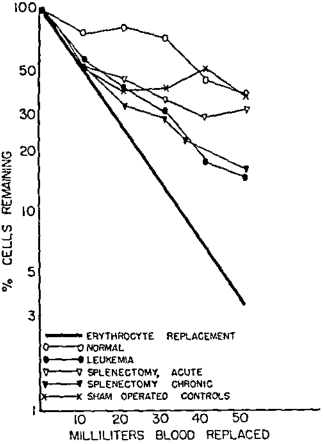

Fig. 4.—Leukocyte changes with massive (50 ml.) replacement transfusions. Leukocyte replacement with leukopenic blood was carried out at rates of 4 to 20 ml. per minute in 5 groups of animals. The mean percentage decrease in leukocyte count for each group during the procedure was compared with the decrease in an adynamic system as typified by the line of erythrocyte replacement (from Hollingsworth, Berend, Silbert, and Finch).[1]

One finds that during the period of cross circulation the recipient maintained an average leukocyte level of only 50 per cent of the donor. This concentration resulted from a net gain of 420 × 10^6 leukocytes during the 80 minute period of observation. The average number present in the leukocyte pool of the recipient, if we accept the value of 3.5 times the blood volume as the immediately exchangeable leukocyte space, was 180 × 10^6, so that enough leukocytes enter the blood to replace completely those contained in the circulating blood nine times per hour or to replace the immediately available leukocyte space 1 75 times per hour, giving an average survival in the leukocyte space of *34 minutes*

When the cross circulation was abruptly terminated and the disappearance of the leukocyte number plotted as a function of time there was a roughly exponential disappearance that had a half time of 27 minutes or an average survival time for leukocytes in the leukocyte space of *39 minutes*, which agrees very well with the 34 minute value obtained above If we accept this value for the immediately available leukocyte pool and use it in the analysis of the surgical parabiosis we find that the average time spent in the leukocyte pool is 3 5 hours, rather than one hour

Because of the rapidity with which these experiments were conducted the *34 or 39 minute values represent the average length of a single sojourn in the* immediately available leukocyte space The average life of a leukocyte in this pool is 39 minutes times the number of sojourns in this pool

If one is willing to combine the data of Hollingsworth and Finch for rapid exchange experiments with the data from slow exchange experiments where equilibrium between the pools is assumed to have been established[3] the analysis can be carried one step further Since the average length of a single sojourn in the immediately available pool was found to be 39 minutes and the total time spent in this pool was found to be 3 5 hours there must be 5 sojourns from the ultimately available pool into the immediately available pool

From the fact that the disappearance of leukocytes after stopping cross transfusion is roughly exponential to 20 per cent of the initial value it is apparent that the leukocytes of the immediately exchangeable pool are a part of a much larger ultimately exchangeable leukocyte space This space has a minimum value of 5 times the immediately exchangeable pool or 17 times the blood volume The ultimately exchangeable pool then constitutes a minimum of ⅘ of the leukocyte space If the leukocyte space has a minimum value of 17 times the blood volume and the average life in the blood is 1 hour then the average life in the leukocyte space for leukocytes as a whole is 17 hours Using the differential count from surgical parabiosis one obtains a minimum mean life for lymphocytes of 170 minutes × 17 or 48 hours and a minimum mean life for granulocytes of 23 minutes × 17 or 6 5 hours

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Discussion

G. Brecher: I think that the most important thing in considering the general problem of cell proliferation is that one has to examine the assumptions very carefully. One of Dr Van Dyke's assumptions is that the exchange of white cells is the same as that of red cells. However, white cells tend to marginate, and I find it rather difficult to accept the idea that in the capillaries, joining parabiotic rats, the white cells will move at the same speed as the red cells. I have seen many photographic records of capillary streaming which have shown a tremendous delay of granulocytes in transit.

The Transfusion of Lymphocytes in Experimental Animals

By J L Gowans

IN ALL the mammalian species so far examined a very large number of cells enter the blood each day from the main lymphatic ducts in the neck Over 90 per cent of these cells are small lymphocytes If the number of lymphocytes in the blood remains roughly constant and if lymphocytes are not rapidly destroyed in the blood (for which there is no good evidence) then large numbers of lymphocytes must leave the blood each day for some unknown destination Thoracic duct cannulation alone shows that the number of lymphocytes entering the blood would replace all those in the blood several times daily in the dog cat rabbit and rat [15] In the rat for example it has been estimated that the combined outputs from all the main lymphatic ducts would replace the blood lymphocytes about 24 times each day [14] If this figure is accepted then rat lymphocytes must spend on the average about one hour in the blood before they migrate into the tissues The problem is to identify the sites in the animal where lymphocytes leave the blood stream and to account for the subsequent fate of these lymphocytes in the tissues [6]

One experimental approach to this problem has been to transfuse labeled or unlabeled lymphocytes into the blood of an animal and then to locate them at various times afterwards in its tissues There is no theoretical reason why an experiment of this kind should not provide the crucial demonstration of the fate of lymphocytes in the animal if the following technical requirements could be fulfilled

1 The cells which are transfused should be those which normally enter the blood and circulate in it that is lymphocytes from the main lymphatic ducts or lymphocytes which have been isolated from the blood A number of workers have studied the fate of suspensions of lymph node cells after intravenous injection Such lymphoid cells have been found in the liver and spleen [1] and in the lymph nodes and bone marrow[3] of the recipient animals Certainly lymphoid cells will function and even proliferate in the tissues of a recipient animal as experiments on the adoptive transfer of immunity have shown [10] However the results which follow the injection of lymphoid cells into an animal may not be due to the activity of small lymphocytes The lymph nodes contain a number of cell types which probably never enter the blood under physiologic conditions and it may be these and not the small lymphocytes whose fate is being observed The results of injecting suspensions of thymus cells[4] are also difficult to interpret since the functional identity of thymocytes and small lymphocytes has never been demonstrated

2 The rate of transfusion should be approximately that at which lymph and lymphocytes normally enter the blood in the rat for example 1 to 2 ml /hr and 20 to 80 × 10^6 cells/hr would be physiologic rates When concentrated suspensions of cells are rapidly injected into a vein or an artery and are found a

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From the Sir William Dunn School of Pathology Oxford University England short time afterwards in the lung[8, 13] and in the kidney,[8] respectively, the first suspicion must be that the capillaries in these organs have been embolized.

3. The transfused cells must be alive when they enter the blood of the recipient animal. Probably the best criterion of viability for lymphocytes is motility in vitro. If the transfused lymphocytes are found in the liver and in the spleen, it must be carefully shown that these organs are not merely clearing the blood of cells which were killed or damaged during their experimental manipulation.

4. Since a transfusion of lymphocytes is, in effect, a homograft, the donor and recipient animals ideally should be genetically identical. In practice, this means that they should be members of the same highly inbred strain. This requirement is essential when the fate of the transfused cells is being followed for many days; it may not be essential for experiments lasting a day or two. Some evidence of a reaction against the donor lymphocytes was obtained in albino rats which had been transfused continuously for four days with thoracic duct cells from a series of hooded rats.[7]

5. The transfused cells should be accounted for quantitatively in the recipient animal. The physiologic turnover of the blood lymphocytes is strikingly large, and it is of little interest in a transfusion experiment if, for example, an odd labeled lymphocyte is found in a particular organ or tissue.

A technic in which an attempt was made to meet these requirements has been described elsewhere.[5, 7] Briefly, thoracic duct lymphocytes were collected from one or more donor rats and were transfused slowly and for periods up to seven days into the blood of other rats of the same highly inbred strain. The cell suspensions were pumped into the femoral vein of the recipient rat at about 2 ml./hr., and the concentration of lymphocytes in these suspensions did not exceed about 40×10^6 cells/ml. The transfused lymphocytes were alive when they entered the rat, since microscopic observation of samples taken from the outlet of the pump showed that the cells were motile. The thoracic duct of both the donor and recipient rats was cannulated by the method of Bollman, Cain, and Grindlay.[2] After operation the rats were placed in restraining cages where they remained, unanaesthetized and in good condition, for periods up to 11 days.

RESULTS OF LYMPHOCYTE REINFUSION AND TRANSFUSION IN RATS

The rapid turnover of the blood lymphocytes is deduced conventionally from the high output of cells during the first day after thoracic duct cannulation. It was found, however, that in order to maintain this high output for periods longer than one or two days, it was necessary to reinfuse continuously into the blood all the lymphocytes which emerged from the fistula.[5] When either cell free lymph or lymph which contained killed lymphocytes was reinfused, a steady fall in the output occurred, similar to that described by Mann and Higgins.[9] It appeared that the continuous entry of living lymphocytes into the blood was in some way essential for the maintenance of the normal output of lymphocytes from the thoracic duct. The mechanism by which the reinfused lymphocytes influenced the thoracic duct output was studied in a series of experiments in which lymphocytes were transfused between members of a highly inbred strain of albino rat.[7] Under a variety of conditions it was found that an intravenous transfusion of lymphocytes resulted in an increase in the output of lymphocytes from the thoracic duct of the recipient animal Thus the low output of lymphocytes which followed the prolonged drainage of lymph from a thoracic duct fistula could be restored to a normal level, the normal level could be more than doubled when a transfusion of lymphocytes was combined with the reinfusion of all the cells *that emerged from the recipient s own thoracic duct* The increase in the output amounted to about 80 per cent of the number of cells transfused It was felt that the extra cells that appeared in the thoracic duct must have originated in one of two ways Either they were new cells which had been formed in the lymph nodes of the recipient animal in response to the lymphocyte transfusion or the transfused cells had recirculated from the blood into the lymph An autoradiographic study of the incorporation of tritium labeled thymidine into the deoxyribonucleic acid of thoracic duct lymphocytes showed that the increase in output was not due to the formation of new cells It seemed likely, therefore that the transfused lymphocytes had somehow found their way back into the thoracic *duct lymph This possibility was tested by transfusing* P^{3} labeled lymphocytes into a rat About 20 per cent of the total radioactivity and 40 per cent of the radioactively labeled lipid originally present in the transfused cells was recovered over a period of four to five days from the cells in the thoracic duct of the recipient rat This experiment was regarded as providing crucial evidence that a substantial fraction of the transfused cells had recirculated from the blood into the lymph The rapidity with which the radioactive lymphocytes appeared in the recipient s lymph together with the high recovery of P^{32} labeled lipid was thought to rule out the possibility of a massive destruction and reutilization of the transfused lymphocytes in the lymphoid tissue of the recipeint It should be added that P^{32} is by *no means an ideal label for experiments of this kind and a* considerable loss of radioactivity from the more rapidly exchanging phosphorus fractions in the transfused lymphocytes no doubt occurred during the four to five days of each experiment

The experimental demonstration that small lymphocytes which are transfused into the blood can be largely recovered in the thoracic duct lymph would provide a satisfactory explanation of the apparently high turnover of lymphocytes in the blood of normal animals The existence of such a recirculation in the normal animal is strongly supported by the demonstration that the rate of formation of new small lymphocytes does not keep pace with the rate at which lymphocytes are discharged from the thoracic duct When tritium labeled thymidine was infused into a rat for the first 12 hours after cannulation of its thoracic duct less than 2 per cent of the small lymphocytes were labeled in each of the four successive 24 hour collections of lymphocytes while almost all the large and medium lymphocytes collected during the first two days after cannulation were labeled[7] In the rat the large and medium lymphocytes make up roughly 5 per cent of the total thoracic duct cells A low rate of new lymphocyte formation was also *found by Yoffey Everett and Reinhardt*[7] *in the guinea pig After a* single dose of tritium labeled thymidine 2 0 to 7 0 per cent of the total thoracic duct cells were labeled over a period of several days A recirculation of lymphocytes is the only hypothesis which can satisfactorily reconcile a high daily output of lymphocytes with a low rate of new lymphocyte formation

The route taken by lymphocytes in passing from blood to lymph is not known

TABLE 1.—*Radioactivity in the Organs of a Hooded Rat (wt. 230 Gm.) that had been Transfused Intravenously with P^{32} labeled Lymphocytes and then Killed 12 hours Later*

	c.p.m./100 mg. wet wt. of tissue
Mesenteric lymph nodes	20
Submaxillary lymph nodes	191
Thoracic lymph nodes	166
Deep Cervical lymph nodes	245
Spleen	148
Thymus	0
Femoral bone marrow	36
Liver	12
Kidney	2
Lung	26
Ileum (including Peyer's Patches)	40
Ascending Colon	46

Total number of cells transfused 8 × 10^8 in 14 hours

Total radioactivity in transfused cells 11 400 c.p.m.

Total radioactivity recovered in recipient's thoracic duct cells up to time of killing 1100 c.p.m.

Lymphocytes were collected from the thoracic duct of two hooded donor rats each of which had received a total dose of 0.5 µc./Gm. body weight of P^{32} as inorganic phosphate. The lymphocytes were washed and then transfused into the femoral vein of another hooded rat of the same inbred strain. Portions of the organs from the recipient rat were weighed and then digested in hot perchloric acid. The radioactivity in the digests was counted in a G.M. liquid counter to give an error of less than 3 per cent.

Sjovall[9] suggested that lymphocytes entered the tissue spaces and returned to the blood via the peripheral lymphatics, but this route probably accounts for only a small fraction of the thoracic duct output.[2, 16] It is possible that the main channel of recirculation may be through the lymph nodes for there is evidence that lymphocytes are added to lymph in large numbers as it passes through a node.[1] Since the experiments with tritium labeled thymidine suggest that these additional cells are not newly formed in the nodes presumably they are extracted by the nodes from the circulating blood. Further support for this idea comes from preliminary experiments in which P^{32} labeled lymphocytes were transfused into the blood of a rat and the radioactivity of its organs determined at various times afterwards. The results of an experiment of this kind are shown in table 1. It can be seen that the lymph nodes and the spleen have concentrated the labeled cells to a greater extent than any of the other tissues examined. In this experiment it was estimated that almost 50 per cent of the total radioactivity which was originally present in the transfused lymphocytes was recovered 12 hours later in the lymph nodes, spleen and thoracic duct cells of the recipient rat.

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Cellular Migration Streams in the Hemopoietic System

By J. M. Yoffey, N. B. Everett and W. O. Reinhardt

IN RECENT YEARS we have been inclining more and more to the view that there may be a number of cellular migration streams between different parts of the hemopoietic system. In the present communication, we discuss three such possible streams, namely (A) *From lymphoid tissue to bone marrow*, (B) *from bone marrow to lymphoid tissue*, (C) *from the thymus to the remainder of the lymphoid complex*. The blood is the common channel through which these migrations occur. The general concept may be presented in a simple diagram (fig. 1).

Stream A. From Lymphoid Tissue to Bone Marrow

The existence of a cell stream from the lymphoid tissue to the bone marrow has been postulated as a result of a number of findings. Among these, one may briefly note (a) The high level of lymphocyte production in lymphoid tissue, (b) the presence of large numbers of lymphocytes in the bone marrow, with no evidence of their having been formed there, (c) the developmental potentialities of the lymphocyte, (d) the negative observation that, apart from the bone marrow, no really satisfactory destination has been suggested for the many lymphocytes that are continually being formed. The interest of the problem is enhanced by the fact that all these findings have been the subject of acute controversy. In the present discussion we shall deal primarily with the high level of lymphocyte production. Other aspects of the problem have been reviewed fully elsewhere.[5, 53, 55, 57]

Estimates of lymphocyte production were based in the first instance on the cells in thoracic duct lymph.[1, 4, 9, 20, 29, 42, 45] Sjovall[45] argued that these cells must be recirculating between blood and lymph, and could not be newly formed. This concept has lately been revived by Gowans,[16, 17] who has extended the observations of Mann and Higgins[30] on rats with prolonged thoracic duct fistula. Yoffey and Drinker,[56] comparing the cell content of peripheral and central lymph, concluded that most of the cells in the thoracic duct lymph of the cat and dog were newly formed. Yoffey, Hanks and Kelly,[5] working with guinea pigs, endeavored to correlate the histologic structure of lymphoid tissue with the quantitative study of lymphocyte output. They also estimated DNA turnover and used this as a measure of lymphocyte production. Both these avenues of approach led to the same conclusion, that for the most part thoracic duct lymphocytes were newly formed cells.[2, 3, 10, 18, 57]

In animals with thoracic duct fistulae the blood lymphocytes, though they never disappear completely, exhibit a marked fall that can be attributed to their leaving the blood for the lymph, which is then drained away via the fistula. In such cases, however, far more lymphocytes can be recovered from the lymph than disappear from the blood. Thus Reinhardt,[41] using unpublished data from the experiments of Shrewsbury and Reinhardt,[44] found that the thoracic duct lymphocytes *could range from 14 to 75 times the number disappearing from*

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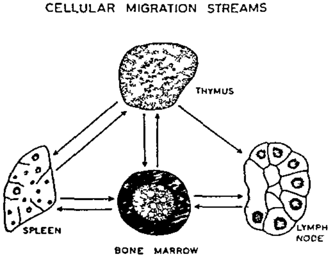

FIG 1—The diagram illustrates the possibilities of cellular migration indicated by arrows (A) From spleen thymus and lymph node to bone marrow (B) From bone marrow to spleen thymus and lymph node (C) From thymus to spleen and lymph node

blood From Gowans data[16] one may calculate that in three rats the thoracic duct lymphocytes ranged from 13 to 49 times those lost from the blood The excess of thoracic duct lymphocytes may be readily understood if most of the cells are newly formed It is also explicable in terms of the recirculation hypothesis if the lymphoid tissues are primarily regions where lymphocytes are stored rather than formed If this view is correct prolonged cannulation should cause *a steady depletion of the lymphoid tissues which should gradually diminish* It would be interesting to see whether this really happens Unfortunately neither Mann and Higgins[30] nor Gowans[16] report on the state of the lymphoid tissues in their experiments

There is further the problem of the normality or otherwise of rats immobilized for many days in the Bollman cage Forcible restraint involving struggling on the part of the animal is a potent method for the production of stress Does the Bollman cage admittedly without obvious violence produce significant degrees of stress and metabolic upset? This is an important point which must be clarified before one can draw any valid conclusions from these fistula experiments It is of course well known that metabolic disturbances can exert a depressant action on lymphoid tissue[55] In the experiments of Gowans[16] the animals appear either not to have gained weight normally during the experimental period or else to have lost weight appreciably

Experiments With Tritium Labeled Thymidine

We have recently performed experiments with tritium labeled thymidine[53] This is believed to be rapidly and specifically incorporated into newly formed DNA[23] and in view of its precise localization in radioautographs[1] seems to be suitable for study of cell formation[11, 4] Guinea pigs were given a single intraperitoneal or intravenous injection of tritiated thymidine and at varying period afterwards both the thoracic duct lymph and the lymphoid tissues were examined

TABLE 1.—*Appearances of Labeled Cells in Thoracic Duct Lymph After Thymidine Administration*

Hours after thymidine	Size distribution formula			Labeled cells per cent
	Large	Medium	Small	
0–1	77	17	0	2.92
1–2	65	29	0	1.83
2–3	58	34	6	3.4
3–4	41	37	15	6.23
14	21	41	35	6.57
72	23	29	36	5.58
144	8	20	69	6.24
192	8	0	92	3.0

The results of one short term experiment (0–4 hours) and four other experiments in which lymph was collected after giving thymidine.

for labeled cells. In table 1 are the results of a short term experiment, and it will be seen that already in the first hour, there are an appreciable number of labeled cells in the lymph. Initially, these are mainly large and medium lymphocytes, and in what we term the SDF (Size Distribution Formula) there are as yet no small lymphocytes. Labeled small lymphocytes begin to appear in the third and fourth hours. After several days (table 1), six to seven per cent of the lymphocytes in the thoracic duct lymph are labeled, and the SDF gradually alters, so that more and more of the labeled cells are small lymphocytes. These thoracic duct data for several reasons do not permit precise quantitation but they do point to substantial new formation of small lymphocytes and are not in accord with the concept of either large scale *recirculation*[17] *or reutilization.*[17a]

Our results in the guinea pig[53] appear to conflict with those in the rat.[17] We can only surmise that this is due either to a marked species difference or to the fact that the rat with a chronic fistula, immobilized in the Bollman cage, is not comparable to the *normal animal.*

The Significance of the Germinal Center

The germinal centers in the lymph nodes also show heavy labeling, indicating that they are regions of active, new cell formation. Not all germinal centers label equally; this is what one would expect from what is known about cyclic changes in them.[8, 31, 57] In some centers, virtually every cell was labeled, which would seem (a) to confirm the cytopoietic function of the germinal center, (b) to rule out recirculation, (c) to rule out also large scale reutilization.[17a]

Sainte Marie and Leblond[42] estimated that in the case of the thymus there were eight generations between the primitive reticular cell and the small lymphocyte. Though we have not been able to make a similar calculation for the other lymphoid tissues, there seems to be no doubt that the broad concept is correct.

Yoffey et al.[67] concluded that, though lymphocyte formation could proceed without germinal centers, the latter constituted a boosting mechanism that came into play when increased production of lymphocytes was required. In addition it may well be that the germinal center is a region of maximal genetic plasticity. Thus when antibody production is stimulated, it is probable that genetically newer strains of cells are produced.[5] It is possible that these new genetic prototypes appear as a result of modifications in the germinal center. Leduc et al[28] have shown that the first evidence of antibody formation is in primitive reticular cell. This does not of course mean that lymphoid tissue is exclusively concerned with antibody formation, or that the germinal centers are specific organs for the formation of gamma globulin.[36] The germinal centers are to be regarded rather as reserves of primitive and undifferentiated cells which normally are concerned with lymphocyte production but which in response to antigenic stimulation can develop in part into antibody forming cells.

Stream B From Bone Marrow to Lymphoid Tissue

The possibility of a cellular stream from bone marrow to lymphoid tissue has come to the fore only in recent years following studies of irradiation damage and protection against such damage by cell transfusion or shielding. In the following paragraph I am assuming that the essential factor in transfusion and shielding experiments is the presence of cells whether or not there is an adjuvant humoral element. The earlier experiments designed to re colonize marrow destroyed by irradiation served to distract attention from the fact that cells from the marrow might re colonize other tissues. They did demonstrate however very convincingly the filtering out of cells from the blood stream into the marrow and the various members of the lymphoid complex. Ford et al[14] using donor cells with an atypical chromosome noted that "Dividing cells containing the marker chromosome have also been identified in spleen, lymph node and thymus." Comparable results with a variety of biologic markings were obtained by other workers.[23, 35, 37]

It was also shown that transfused splenic cells could colonize bone marrow and lymph nodes.[23, 37] But did the seeding of artificial cell suspensions have any counterpart in the intact animal? Jacobson[19] and Kaplan[21] showed that merely shielding the spleen gave rise to earlier and better regeneration of marrow and lymphoid tissues. It should perhaps be noted that the spleen in the smaller laboratory animals usually contains erythropoietic and occasionally granulopoietic foci.

A number of observers found that transfusion of marrow cells into irradiated animal stimulates the regeneration of lymph nodes and thymus.[7, 12, 19, 36, 48] Here too the problem arise of whether cell can normally stream from the marrow to the lymphoid tissues the thigh shielding experiments of Kaplan and Brown[22] and the shielding of ectopic marrow by Storer[46] seem to indicate clearly that such must be the case. Nagareda and Kaplan[34] further found that shielding the thigh of hypophysectomized mice during irradiation effectively promoted complete regeneration of lymphoid tissues.

Kaplan et al[4] endeavored to identify the protective agent in bone marrow and concluded that it was closely associated with marrow cell highly radiosensitive and less effective when given intraperitoneally. All this seems to point to actual cells. However they cite the work of Cole[6] which suggested the possibility of subcellular fragments being involved. But since the material to be effective *must be made available in relatively large quantities* and is not highly potent[24, 29] and since there appears to be a quantitative relationship between the number of marrow cells transfused and the speed and extent of thymic regeneration[49] it seems that in all probability it is actual cells and not cellular fragments that are responsible for the protective effect If the protection is conferred by actual cells the question which at once arises is What is the cell concerned? In a suspension of bone marrow, a possible cell to consider would be either the primitive blast cell or the reticular cell The primitive blast cells are few in number[5] and the reticular cells still fewer[54] From what we know about the cells in guinea pig marrow[51, 53] the most numerous cells and also those most likely to be responsible for the recolonization of lymphoid tissue and thymus are the small lymphocytes and the transitional cells That these should in part colonize thymus and lymph nodes after the intravenous transfusion of marrow suspensions is not too difficult to accept But if they do so after marrow shielding *the implication is that numbers of these cells can normally leave the marrow*

In previous studies[56, 58] the assumption has been that the large lymphocyte population of the marrow is relatively static that lymphocytes stream to the marrow from the blood and remain there An analysis of the various transfusion and shielding experiments suggests that this is possibly an oversimplification It may well be that the level of lymphocytes in the marrow is a dynamic rather than a static equilibrium and that there is a constant turnover of the marrow lymphocytes This could have the effect of making the marrow a large and readily available lymphocyte reserve from which lymphocytes can be drawn much more speedily than the formative lymphoid tissues where time is required for their formation whereas in the marrow they are not formed but stored and are available for immediate delivery Once however the lymphocytes in the marrow *begin to grow and differentiate they are then withdrawn from the mobile pool*

One additional finding may be adduced in support of this concept It has been noted elsewhere[53] that considerable numbers of lymphocytes may be found in the marrow sinusoids a phenomenon described as lymphocyte loading If as has been suggested the flow of blood through marrow sinusoids can be intermittent *lymphocytes could accumulate in stagnant sinusoids and be immediately* swept into the circulation when the flow of blood is resumed

Stream C From Thymus to Remainder of Lymphoid Complex

The relation between the thymus and the remainder of the lymphoid complex has been the *subject of speculation for many years*[55] Kindred[7] *on the basis of* mitotic counts in 15 and 20-day old albino rats concluded that the thymus is the most active producer of lymphocytes in the body, and that its lymphocytes help to maintain the lymphocyte population of the lymph node This of course would mean a constant cell stream via the blood Andreasen and Christensen[2] *found that in young animals the thymus had four to six times as many mitoses* as did lymph nodes Andreasen and Ottesen[3] found that the turnover of DNA was two to five times as active in the thymus as in other lymphoid organs The *picture thus is one of active cell proliferation in the thymus with cells presumably* entering the blood and settling in other lymphoid tissues If this is so then the thymus must be regarded as the pacemaker of the lymphoid organs the leader of the lymphoid complex Is there any evidence to support this concept? McEndy et al[29] have shown that thymectomy markedly diminishes the occurrence of spontaneous lymphomata in certain high leukemia strains of mice Kaplan[21] found that in C57 black mice the incidence of radiation induced lymphoma was virtually abolished by preliminary thymectomy Furth and Boon[15] were of the opinion that the thymus acted as a source of malignant cells but that removal of the thymus had no systemic effect apart from this The fact that tumors may develop in a thymus grafted from a normal to an irradiated thymectomized animal[25] indicates that the thymus itself may be influenced by extrathymic possibly cellular factors

If the thymus is continually producing cells to replenish the lymph nodes then thymectomy should result in appreciable changes in these nodes with diminished lymphocyte output in consequence It was from this angle that we ourselves approached the problem[40] The thymus was removed in young male guinea pigs which reached a weight of approximately 400 Gm 40 days after operation *Whereas in 10 control animals the mean hourly output of thoracic* duct lymphocytes was $18\ 9 \pm 3\ 7 \times 10^6$ 40 days after thymectomy the mean hourly output in seven animals was found to be $8\ 4 \pm 1\ 3 \times 10^6$ This was an unexpected result for we had anticipated that a compensatory hypertrophy of the remaining lymphoid tissue would maintain lymphocyte production at its pre thymectomy level The diminution in lymphocyte output after thymectomy could readily be explained on the assumption that a stream of cellular reinforce*ments from the thymus to the lymphoid tissues had been cut off* Such an explanation would imply that even without these reinforcements lymphocyte formation in the lymphoid tissues could continue though at a reduced rate It could be argued however that the thymus supplies some hormonal factor such as the lymphocytosis stimulating substance of Metcalf[32] in the absence of which lymphocyte formation is diminished One would have to assume then that even without such a factor a fair degree of lymphocyte formation could take place

Fichtelius[9, 10a] transfused cell *suspensions from animals that had been given* P^{32} and followed the fate of the labeled cells in the recipient animals He concluded that the spleen receives from the thymus a stream of cells which act as plasma-cell precursors Plasma cells are not normally formed in the thymus itself

Thymic Depletion

The changes in the thymus following *irradiation may serve as another possible* approach to the problem Kaplan and Brown[23] noted that in mice recovering from irradiation the weight of the lymph nodes recovers slowly whereas thymic weight rebounds vigorously to an abortive peak at about 10 days There is then a more gradual but sustained recovery which may require as long as 70 to 80 days to reach the control baseline The secondary sharp decrease in weight is not associated with histological evidence of a new wave of cellular radiation damage The explanation of this bizarre oscillation must await further experimental study It is interesting however that no secondary involutional phase occurs when thymic regeneration is promoted by spleen or thigh shielding or bone marrow injection

The fall in thymic weight without sign of damage could be attributed to excessive depletion of cells that are needed elsewhere, probably both in lymphoid tissues and bone marrow Harris[18a] has shown that in guinea pigs recovering from irradiation, the marrow takes up enormous numbers of lymphocytes during the first fortnight or so of the recovery period If the other members of the lymphoid complex are also rapidly removing lymphocytes from the blood stream at the same time, one could readily understand the thymic depletion and loss of weight, without the organ damage Though this explanation would fit in with the observed facts, it is difficult to conceive of a mechanism by which the escape of cells from the thymus could be controlled

The view that the thymus is depleted by the heavy demand for cells receives further support from the final observation quoted from the paper of Kaplan and Brown[23] Animals whose recovery was promoted by thigh or spleen shielding or by injection of marrow cells, did not show secondary thymic involution These procedures would doubtless promote earlier and more complete regeneration of lymphoid tissue, and bone marrow, and in consequence lessen the need for large numbers of cells from the regenerating thymus

Summary

The blood cells can migrate from one part of the hemopoietic system to another Three migration streams are proposed for the lymphocytes, namely (A) From the lymphoid tissues to the bone marrow, (B) from the bone marrow to the lymphoid tissues, (C) from the thymus to the remainder of the lymphoid complex

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Discussion of Lymphocyte Recirculation

L D HAMILTON One point of Dr Gowans' work deserves emphasis After 24 hours only a small proportion of the small *lymphocytes, which constitute 95 per cent* of the lymphocytes, were labeled It is important to remember that if only 5 per cent of the total lymphocytes are labeled, this may represent almost 100 per cent of one cell type There seems to be no clash at all between Dr Yoffey's and Dr Gowans' figures, both indicating quite a long life span for the small lymphocyte

N B EVERETT One point on which I would like to seek counsel with you gentlemen is the problem of the weak labeling of the small lymphocytes several days after thymidine administration If we take lymph smears, six to eight or more days after thymidine administration, we get a much larger number of lymphocytes labeled most of which are small, weakly labeled cells (Discussion fig 1)

J L GOWANS I should have thought that Dr Yoffey's figures[1] for the proportion of thoracic duct lymphocytes which incorporate tritiated thymidine do not support the idea that the majority of the small lymphocytes in the thoracic duct of guinea pigs are newly formed cells If only 2 to 7 per cent per day of all thoracic duct cells are labeled for several days after a single injection of tritiated thymidine, then one wonders where the other 90 per cent or so come from Assuming that the period of DNA synthesis occupies a substantial part of interphase, a single injection of tritiated thymidine would be expected to label a considerable fraction of a single day's output of lymphocytes if this output were maintained by the continuous formation of new cells The 2 to 7 per cent of total labeled cells presumably includes during the *first day at least, the medium and large* lymphocytes which, we all agree, incorporate tritiated thymidine very rapidly This reduces to rather small proportions the rate of production of small lymphocytes

It has been suggested that in the guinea pig the magnitude of the DNA turnover in the lymph nodes is sufficient to account for the observed output of cells from the thoracic duct, and that this fact makes a large scale recirculation of lymphocytes in the guinea pig very unlikely There are two points I should like to raise here First, I am not clear how studies on the incorporation of P^{32} into the DNA of lymph nodes can give any reliable information about lymphocyte production Second, the figures for thoracic duct lymphocyte output in the guinea pig are based on cutting the duct in an anesthetized animal and collecting the lymph with a pipet for a total period of two to three hours[2] In the rat a single two- to three-hour lymph collection would give a very inaccurate indication of the actual output of cells when compared with estimates based on 24 hour collections of lymph This is particularly true for a two- to three-hour collection taken immediately after the operation The guinea pig and the rat may differ in the extent to which the thoracic duct lymphocyte output is maintained by a recirculation of cells from the blood, but the data for the guinea pig, in my view, do not allow this conclusion at present

Finally there is the problem of the fate of the large and medium lymphocytes In the rat, the output of these cells falls only slightly on prolonged drainage of lymph from a thoracic duct fistula This together with the fact that virtually all the large and medium lymphocytes in rat thoracic duct lymph become labeled when tritiated thymidine is infused intravenously for 12 hours, makes it very likely that their output is maintained in the rat by the continuous formation of new cells in the lymph nodes I do not think it can yet be decided whether the larger lymphocytes turn into the small or whether the two classes have independent precursors and independent fates

J M YOFFEY If small lymphocytes don't come from large and medium lymphocytes where do they come from?

In replying to the point which Drs Gowans and Hamilton made, I think it is a little fallacious to say, "Ah only 6 per cent of small lymphocytes are labeled and therefore the rest of them must be recirculating" I would leave it to the thymidine pundits like Dr Quastler, to give you the full story However, if you give a single dose of thymidine, it is taken up in a matter of 10–15 minutes, but it only labels a small part of the growing population—

(FROM THE FLOOR Over half depending on the cell population)

J M YOFFEY Take it as 50 per cent, which would double the figure, though the studies of Firket[3] seem to throw doubt on this The other more important point is that thymidine makes computation difficult because you get cells liberated over many days from a precursor pool If you could compress the release over a short time you would get a much truer idea of the actual new cell output

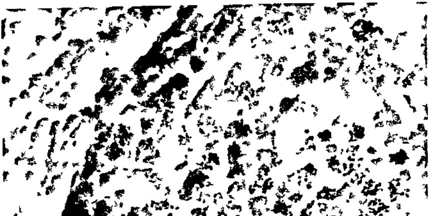
Finally nobody disputes that there is some recirculation of lymphocytes Dr Drinker and I, trying by what we thought then was a convincing approach (namely, cannulating peripheral lymphatics and studying the cell content of peripheral lymph), observed that there certainly were some small lymphocytes recirculating but we calculated that they were only a small percentage of the total, between 3 and 5 per cent There may conceivably be an even higher percentage recirculating in the rat But now it is quite clear to us, on the basis of examination of our guinea pig material containing many germinal centers in

DISCUSSION FIG 1—Radioautograph of thoracic duct lymph from guinea pig six days after receiving 1 μc/Gm of H^3 thymidine

which practically every cell takes up DNA, that there is very substantial new formation of lymphocytes (Discussion fig 2) To suggest that the bulk of them are recirculating all the time just does not fit in with these findings.

S PERRY In this regard we did the following The thoracic duct of a dog was cannulated lymphocytes collected labeled with P^{32} washed and then reinfused The dog was followed for as long as three days and labeled cells were never seen in the thoracic duct The transfused cells were viable, or appeared viable on supravital staining and phase microscopy We feel that there were a sufficient number of cells infused so that they should have been detected in the thoracic duct

J AMBRUS I was very much surprised to hear Dr Perry's results We did a similar experiment However, instead of labeling the lymph *in vitro after collection*, we treated the donor dog with fractional doses of P^{32} for several days prior to collection of lymph Following infusion into recipients, the specific activity of DNA in the lymph was analyzed Under these conditions we found very significant lymph recirculation A similar experiment was done with tritiated thymidine When in vitro labeling technics were used, large numbers of large lymphocytes were labeled They did not recirculate We found a few in bone marrow and lymph nodes On the other hand if we pretreat a dog for several days with fractionated doses of



DISCUSSION FIG 2—Radioautograph of an active germinal center from the superior mesenteric lymph node of the guinea pig four hours after the intravenous administration of 1 μc/Gm of H^3 thymidine × 606

tritiated thymidine and produce some labeling of the small lymphocytes we see some small lymphocytes recirculating

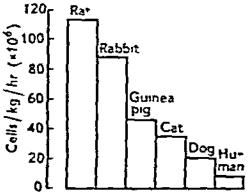
We have labeled in vitro human large lymphocytes collected from thoracic duct cannula These did not seem to recirculate to any appreciable extent following intravenous infusion although we did see a few in bone marrow So I suspect that there is a *significant recirculation of small lymphocytes* However most of the large ones get caught either in lymphoid tissue or bone marrow

J M YOFFEY There is quite a graduation in the thoracic duct lymphocyte output of a series of animals (Discussion fig 3) It may well be that in the rat which has the highest output in the series there is much more recirculation and less true formation At the other end of the scale is man, with apparently the lowest lymphocyte output of all. This also applies in comparing bone marrow lymphocytes in man with those of the lower animals, where the lymphocyte population may be much higher. So that is the first caution. One must be very careful before contradicting with data from another animal.

A second point concerns a rather more careful analysis of the recirculation problem. There are one or two gaps here and I won't be happy about even attempting to interpret experiments like those of Dr Gowans until these gaps are filled. The recirculation hypothesis postulates that lymphocytes pass from the blood into the lymph stream through the lymphoid tissues and into the lymph of the thoracic duct. Now, if you take a system like that, cannulate the thoracic duct, on the basis of Dr Gowans' own calculation the blood should be depleted of its lymphocytes in the course of two hours. Now that doesn't happen, and for 24 hours you get pretty *much the same lymphocyte output through* the thoracic duct, so that it is more involved than simple recirculation.

As I see it, this can only mean that a lot of lymphocytes must still be entering the thoracic duct from lymphocyte reserves. *If Dr Gowans is right* about the nonformation of small lymphocytes in the lymphoid tissues, one would have to regard these solely as lymphocyte depots, and over a period you would get a gradual depletion of the lymphoid tissues.

J L GOWANS: I should like to comment on Dr Yoffey's remarks about the difficulty of accepting the idea of a large scale lymphocyte recirculation. My own views are based on experiments in the rat which I *interpret* as showing that lymphocytes which are transfused into the blood can be largely recovered in the thoracic duct lymph. When P^{32} labeled lymphocytes were transfused, the radioactivity was localized predominantly in the lymph nodes and the spleen at a time when radioactive cells were appearing in the thoracic duct lymph. This suggests that one important route of recirculation from blood to lymph may be via the lymph nodes. Certainly the lymph nodes *of rats with long standing* thoracic duct fistulae look *depleted of* small lymphocytes.



DISCUSSION FIG 3.—Comparison of figures for thoracic duct lymphocyte output in different animals. (From Yoffey and Courtice [1956] fig 83 p 363. We are indebted to Dr W O Reinhard for this figure.)

In the rat, almost all the thoracic duct lymphocytes originate from the bed drained by the intestinal lymphatics.[4] Thus, if a massive recirculation of lymphocytes occurs, the main channel from blood to thoracic duct lymph must be somewhere in the splanchnic area, either in the lymphoid tissue *in this* area *or in the gut itself*. A recirculation through the peripheral lymphatics of the gut is not excluded by the experiments of Yoffey and Drinker[5] in which very few cells were found in lymph collected from *hind limb* peripheral lymphatics.

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Section II. DNA Synthesis Stability and Degradation

E Chargaff, *Chairman*

The Metabolic Stability of Deoxyribonucleic Acid

By Walter L Hughes

IT WOULD APPEAR that my intended function in this paper is to set a background of well being So that while individuals may still argue about the identification of cells and their spatial and temporal relationships nevertheless it must all be chemistry and thus by referring everything to the fundamental and incontrovertible deoxyribonucleic acid (DNA) a way out of the morass will be found *However at this level too there is quicksand And I have chosen to talk on the touchy theme Does DNA turn over?*

Evidence that DNA metabolism is quite different from that of most macromolecules is readily obtained from a variety of sources Thus autoradiography of a tissue culture labeled with a protein precursor gives a very diffuse distribution of label On the other hand one labeled with the DNA precursor thymidine shows label confined to the nuclear region Furthermore only a few nuclei are labeled This shows that DNA synthesis is not proceeding continuously in all cells

Now it is frequently suggested that protein is made by RNA and RNA is made by DNA so who makes the DNA? Until recently this question was like Which comes first the chicken or the egg? However recent work has made the answer obvious Kornberg makes the DNA

BIOSYNTHESIS OF DNA

Kornberg's group in a recent series of publications has mapped out a simple *biochemical system for DNA duplication*[1,2] [3] *Perhaps in keeping with the fundamental role of DNA but certainly at odds with its complexity this system* can be simplified to a few components all soluble under physiologic conditions Kornberg's studies have been primarily on bacteria therefore this discussion will touch only the high points Dr Potter in a later article may supply more details in discussing his more pertinent mammalian system

Kornberg's group has shown that when a mixture of the four natural deoxynucleoside triphosphates are incubated with magnesium ions and an enzyme from *E coli* in the presence of deoxynucleic acid as a primer all four of the nucleosides are fixed to the DNA with the liberation of pyrophosphate

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$$\left.\begin{array}{c} n\ \mathrm{dAPPP} \\ + \\ n\ \mathrm{dGPPP} \\ + \\ n\ \mathrm{dCPPP} \\ + \\ n\ \mathrm{TPPP} \end{array}\right. + \mathrm{DNA} \rightleftharpoons \mathrm{DNA} - \begin{bmatrix} \mathrm{dAP} \\ \mathrm{dGP} \\ \mathrm{dCP} \\ \mathrm{TP} \end{bmatrix}_n + 4(n)PP$$

Enzymatic degradation shows that these are in fact linked as 3 5-diphosphate esters through the sugar in the normal fashion of polydeoxynucleotides. Furthermore this is not an exchange reaction but true synthesis and with their most purified enzyme large amounts of net synthesis have been observed. Table 1 shows that not only are large amounts of radioactivity bound but that also there *can be a 2 to 20 fold increase in the optical density of the material precipitating* as DNA.[2] If any one of the four bases is omitted from the reaction mixture no net synthesis occurs and only a trace of radioactivity is added to the DNA. This trace is approximately equivalent to the addition of one nucleotide per molecule of primer DNA and in fact all the added radioactivity can be shown to be at the end of the DNA chain.

These findings agree well with the base pairing of the nucleotides proposed by Watson and Crick[20] as a result of x-ray crystallographic studies on DNA. A further test of this hypothesis in the isolated synthetic system by substitution of analogues of the natural bases has produced substitutions in the synthesized DNA[3] but only those permitted by the hydrogen bond requirements of the Watson-Crick scheme. An outgrowth of this is their finding that deoxyuridine may be incorporated into DNA in vitro. The reason why this does not happen in *vivo is due to the lack of an enzyme capable of triphosphorylating deoxyuridylate*.[3]

STRUCTURE OF DNA

Utilizing this concept of base-pairing Watson and Crick have been able to interpret the crystallographic data in terms of a double helical structure for DNA.[6] The details of this structure pertinent to the present discussion are (1) The DNA molecule is composed of two long polynucleotide chains coiled about each other. (2) These chains run in opposite directions. (3) The purine and pyrimidine bases do not dangle but are neatly packed within the coil and held in place by specific pairs of hydrogen bonds. (4) All of this gives the molecule a

TABLE 1 *Net Synthesis of DNA*

Experiment No	Estimation	Control no enzyme	Complete	Δ µmoles
1	P³² incorporation	000	280	280
	Optical density	193	458	265
	Deoxypentose	*18*	*395*	*208*
2	Optical density	060	628	568
3	Optical density	052	583	531
4	Optical density	051	642	591
5	Optical density	041	889	848

high degree of rigidity and inflexibility, so that DNA, like many proteins, denatures irreversibly.

DNA REPLICATION

Now, how *does the actual replication take place? Fortunately, data on this* process are accumulating, and again it appears to occur in the simplest possible way. Meselson and Stahl[14] produced heavy DNA by growing *E. coli* in a glucose salt medium with N^{15} ammonia as the sole source of nitrogen. Then they followed the fate of such labeled DNA as a result of further growth in a medium containing an excess of the light N^{14} isotope.

To do this they developed a very ingenious ultracentrifugal technic. If a macromolecule such as DNA is dissolved in a concentrated solution of cesium chloride of density approximating that of the DNA, and ultracentrifuged, the centrifugal force tends to sediment the cesium chloride, producing a density *gradient. Consequently, the DNA molecules tend to seek out the region in the solution of equal density,* and after sufficient time with sufficient centrifugal force, all the molecules collect in a band. The width of this band is, of course, determined by diffusion and hence is inversely related to the molecular weight. The beauty of this method is that very small density differences may be determined with minute amounts of material. Furthermore, since other types of macromolecules, such as proteins and even RNA, have very different densities, they may be floated or sedimented completely away from the DNA.

Figure 1 shows the results of a series of analyses of samples of DNA obtained *from bacteria labeled with heavy nitrogen after various numbers of duplications in the presence of light nitrogen.* Notice that *after only a few minutes of growth* a new and distinct band of lighter DNA is formed, with the concomitant disappearance of the original heavy DNA, which is complete after one generation. During the second generation a new, still lighter, DNA appears, but no longer at the expense of the heavier component. With still further growth these two bands remain, the heavier becoming proportionately but not absolutely smaller. By comparison with the last analysis, which is of a mixture of normal and N^{15} DNA, it can be seen that the light band corresponds precisely to normal N^{14} DNA, *and the intermediate band has a density just half way in between and* therefore must be composed of equal amounts of light and heavy DNA.

Further proof of the hybrid nature of this intermediate DNA was obtained by heat denaturation. Density gradient centrifugation following this treatment (fig. 2) showed the single band to have been replaced by twin bands whose molecular weight was half the original value. Furthermore, their densities were identical with denatured N^{14} and N^{15} DNA, respectively.[14]

A mechanism of duplication consonant with these results and with the double stranded helical structure is shown *in figure 3. This suggests that the DNA molecule is self complementary, each helix being able to act as a template for the* formation of its other half. Thus, Meselson and Stahl have found at the molecular level the mechanism of duplication for the genetic material which we had previously found at the chromosomal level.[15]

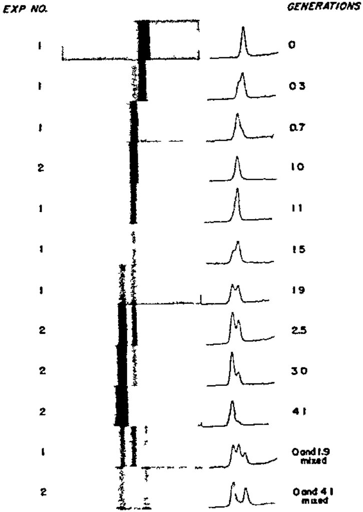

FIG 1—Ultraviolet absorption photographs (left) showing DNA bands resulting from density gradient centrifugation of lysates of bacteria Center column records microdensitometric measurements of the photographic plates Generations indicates the fraction of the generation time during which the culture of N^{15} labeled *E coli* was allowed to grow in N^{14}-containing medium before sampling (Reproduced from Meselson and Stahl *Proc Nat Acad Sci* by permission [14])

CHROMOSOMAL REPLICATION

I will now quickly sketch our evidence[15] [18] We chose tritium as the radioactive indicator because of the excellent autoradiographic resolution it provides, and thymidine as the carrier because of its specific utilization for DNA synthesis The thymidine was tritiated by exchange from tritium acetate with reduced palladium black as a catalyst (fig 4) After purification, by repeated exchange with water to remove loosely bound tritium, and chromatography it had a specific activity of about 300 curies per mole

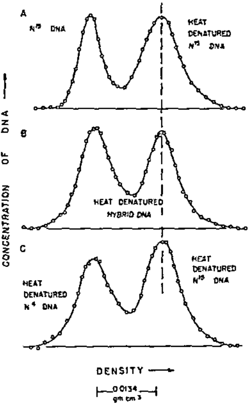

FIG 2 —The dissociation of hybrid DNA upon denaturation Microdensitometric tracings as in figure 1 (center) (A) A mixture of heated and unheated N^{15} DNA from bacterial lysates *Note that denaturation increases the density and also increases the band* width indicating dissociation to a smaller molecular weight (B) Heated denatured hybrid DNA The single band in the fourth photograph of figure 1 is now replaced by two bands *of densities equal to those of denatured N^{14} and N^{15} DNA shown in (C) (Reproduced* from Meselson and Stahl *Proc Nat Acad Sci* by permission [14])

Vicia roots were chosen for the biologic system because of previous information as to their mitotic cycle Roots were grown in the labeled thymidine solution for *eight hours resulting in the labeling of about one third of the cells Then they* were transferred to colchicine solution and samples taken for autoradiographic analyses at subsequent intervals The colchicine prevented nuclear division, but not chromosomal duplication Consequently, any labeled mitoses with double the normal number of chromosomes must have carried out an additional division after removal from the labeling solution

Chromosomes at *metaphase in labeled diploid nuclei* (fig 5) were always uniformly labeled along both daughter chromatids indicating that all new chromosomes contain new DNA However after longer intervals in the colchicine solution when tetraploid mitoses appeared (fig 6) only one of each pair of daughter chromatids was labeled Therefore, the duplicating unit of the cell must be a half *chromosome, and duplication* (fig 7) *consists in the separation of the complementary* halves of chromosomes followed by the synthesis of new complements [15]

We have also found the same mechanism in bacteria [16] A thymine requiring mutant of *E coli* was labeled during several generations growth in H^3 thymidine,

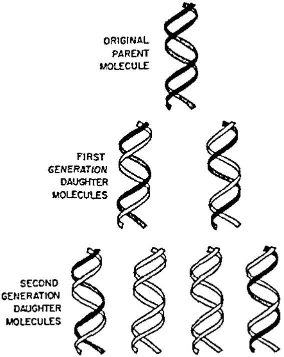

Fig 3 —The mechanism of duplication of DNA as proposed by Watson and Crick Heavy and light strands of DNA as measured in the present studies are indicated by shading Each daughter molecule contains one new and one parental strand so that the distribution during successive replications would be as found (fig 1) (Reproduced from Meselson and Stahl *Proc Nat Acad Sci* by permission [14])

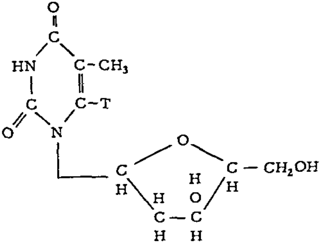

Fig 4 Tritiated thymidine

so that all the DNA was labeled Samples were then analyzed autoradiographically after further growth in nonradioactive medium After one generation in nonradioactive medium all cells were still labeled, but with only half the intensity of the inoculum However, by the third generation, large numbers of un

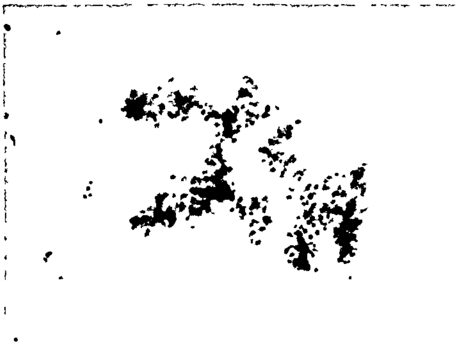

FIG 5 —Autoradiogram of Vicia chromosomes at metaphase immediately after duplication with labeled thymidine, showing uniform distribution of label

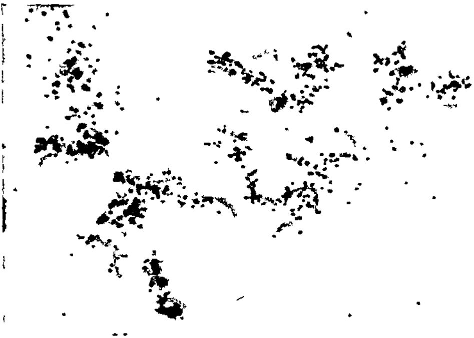

FIG 6 —Autoradiogram of second duplication of labeled chromosomes in the absence of labeled thymidine showing labeled and unlabeled chromatids

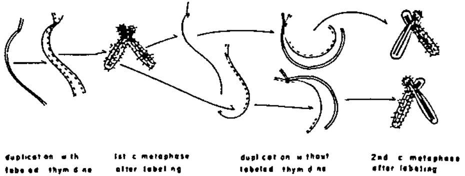
duplication with labeled thymidine
1st c metaphase after labeling
duplication without labeled thymidine
2nd c metaphase after labeling

FIG 7 —Diagrammatic representation of mechanism of chromosomal duplication Dashed lines indicate formation and subsequent distribution of labeled DNA through two chromosomal duplications

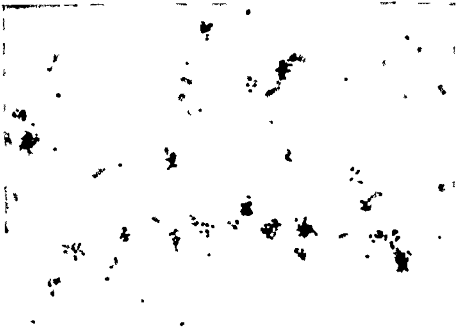

FIG 8 —Autoradiogram of *Escherichia coli* 15 T labeled by several generations growth in tritiated thymidine and then grown for about three generations in nonradioactive medium Note that some cells are unlabeled while in other cases the silver grains are so numerous as to obscure the bacterial cell

labeled bacteria had appeared (fig 8) This indicates that the nucleus of this organism must be a single chromosome, which also duplicates and is transmitted as half-chromosomal units

The support which all of these different lines of evidence lend to each other makes one quite confident of the essential rightness of the over all concept, although refinements and second-order approximations will doubtless prove necessary all along the line However, there are still many parts of the problem about which nothing is known Some which appear particularly pertinent to our dis cussion are What holds the DNA molecules together in the chromosome? Is it protein? Is it also transmitted intact to the progeny? What starts and stops DNA synthesis? How does DNA function? What is a gene?

Does DNA Turn Over Within the Cell?

To return to a more limited objective what bearing does all of this have on the use of labeled DNA in following cell proliferation? Is a cell making DNA always preparing to divide? I believe this will turn out to be the usual case Painter is labeling DNA to measure the elapsed time between synthesis and mitosis in tissue cultures of HeLa cells This time is not constant since three hours after the introduction of the label both labeled and unlabeled mitoses are found However by six to eight hours all mitoses are labeled indicating that the variation in this period is only moderate However one has only to consider polyploidy and polyteny to realize that cell division does not always follow DNA synthesis Dr P Woods at Brookhaven has used H^3 thymidine to label the polytene chromosomes of the salivary glands of drosophila[21] Labeling begins at an early larval stage and continues until the chromosomes have matured into tremendous structures containing hundreds of times the normal amount of DNA

But leaving out synthesis of extra DNA can DNA be labeled by exchange processes? Obviously these do not fit into the scheme outlined Studies on synthesis show no evidence for exchange reactions Moreover Kornberg's group has been unable to reverse the synthetic reaction which may only mean that the equilibrium is very unfavorable

However what about accidental synthesis when the signal has not been given? This question cannot be answered from our present knowledge of the synthetic system but the concept is hard to reconcile with a precise mechanism for the maintenance and transmission of genetic material

Finally what about exchange? This can be easily visualized only at the end of the polynucleotide chain and since these chains contain thousands of mononucleotide units only a tiny fraction of the amount of label introduced by synthesis could be so attached and this amount is not likely to be detected autoradiographically On the other hand Zamenhof has claimed that large amounts of bromouracil exchange for thymidine in resting cultures of *E coli*[22] If this does occur it must be a unique property of his mutant since *E coli* is the very source of Kornberg's polymerase and his group observes no exchange More likely what Zamenhof calls exchange is actually continuing synthesis in a population of cells where death balances birth

However alternate mechanisms of labeling certainly cannot be ruled out by armchair research The systems thus far described are not necessarily sensitive enough to rule out other mechanisms Therefore perhaps the most precise answers to the exchange question will come from studies of labeled cells In this regard Painter's studies in tissue culture indicate that cells may be either heavily labeled or completely unlabeled (fig 9) Thus if exchange takes place in this system it must be intermittent Such rapid exchange does not seem to be postulated at the present time Slow exchange following a single administration of isotope will probably not confuse the analysis of cell populations since the labeling precursor will not be present long enough However reverse slow exchange

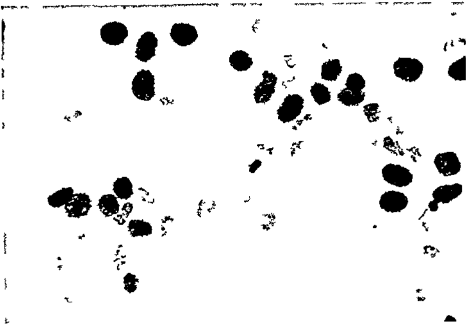

Fig 9—Autoradiogram of a culture of HeLa cells grown for a few minutes in tritiated thymidine (Feulgen stained)

can give false estimates of cell survival This in turn then seems to suggest the most critical test for exchange the comparison of cell life span as measured by several labels

Reassurance as to lack of turnover of DNA has come from several studies of labeled cells in continuous culture Throughout ten or more generations the label in DNA has been retained quantitatively in the whole cell mass [4, 5, 17] However these studies also showed that RNA was equally conserved and this is certainly at variance with whole animal studies

Davidson s group has reinvestigated this phenomenon and obtained data that markedly weaken the former evidence [9] They operated in slow growing tissue cultures and showed that label was rapidly lost from RNA while being largely but not completely retained in DNA Thus the error seems to be that the interior of rapidly growing cells is not in equilibrium with the environment and the breakdown products of RNA catabolism are immediately seized for new RNA synthesis within the same cell Consequently the previous tissue culture evidence for DNA stability is inconclusive However equally inconclusive is any evidence for DNA turnover based on small losses of DNA unless cell death can be excluded

Long term retention of label in DNA with simultaneous elimination of label *from RNA has also been observed in bulk tissue studies on mammals Dr Potter s* group has provided excellent data on the stability of DNA in regenerating liver [6, 7] and it is hoped that he will comment further However we cannot assume that DNA in all cells will behave similarly Certainly Dr Bendich can provide evidence that all DNA is not identical and this may be related to different sources of origin (different cells) in the same tissue

Recent publications by Pelc[16] and by Koenig[10] serve to re-emphasize this issue They claim to have shown autoradiographically extensive uptake of C^{14} into the DNA of the nuclei of mouse seminal vesicles and the feline neuraxis, respectively Since they used adenine as a precursor, RNA was heavily labeled, and their evidence rests on radioactivity not eluted by the action of cold perchloric acid or RNase, but eluted by hot perchloric acid or DNase

Koenig suggests that this surprising result may stem from the precursor used Since RNA was also labeled, its continuous turnover maintained a high level of labeled precursors for both RNA and DNA within the cell's metabolic pool Consequently, significant incorporation into DNA was possible in spite of the very slow turnover rate postulated by Pelc

Before accepting these experiments, it is important to ask whether they proved that DNA was labeled No criteria for purity or specificity of the enzymes were given, and evidence for a quantitative separation was inferred from studies on other tissues It seems quite possible that extraction conditions may vary with the tissue studied, and some RNA might remain bound in the nucleus, being eluted only after the destruction of DNA If further work confirms that the DNA in these cells is labeled, can this only be interpreted as exchange? Is it certain that these cells are not proliferating, and if this be so, is it certain that they do not contain extra DNA?

CONCLUSION

In view of the strong and mutually corroborating types of evidence for lack of turnover of DNA, I think that the burden of proof is on the discoverer each time a new system exhibiting exchange is reported He must first prove that the label is, in fact, in DNA, and having proved this, he must show that the label is not introduced as a result of synthesis, i e , that the DNA content of the cell in question remains constant during the labeling period

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Discussion of DNA Stability

L G LAJTHA I should like to mention some of Dr Pelc's experiments which have some relevance to the problem of turnover or stability of DNA [1,2] He finds that in the lower meristematic region of the bean root (vicia faba) where the growing cells occur the DNA *content varies from 2n to 4n* If he incubates such roots with thymidine he gets a certain grain count let us say about 30 grains per labeled cell Above that region however, there are rows of elongated cells which don't incorporate P^{32} or adenine C^{14} i e they don't synthesize DNA so far as we know They contain a remarkably constant 4n amount of DNA without transitional values and without active DNA synthesis However when the roots are incubated with thymidine he finds large proportions (whole rows) of these elongated cells labeled with thymidine and having 60 grains per cell This I am afraid would *mean on the face of it that the whole* tetraploid amount of thymidine is released and exchanged with new thymidine

Also when Dr Pelc's group repeated some of the chromosome duplication experiments of Taylor et al [3] they found that if they omit colchicine treatment they do not get the Taylor type of chromosome duplication but if they use colchicine they get exactly the same results as Taylor They wondered if all this might not indicate an artifact due to colchicine

W L Hughes So far as the second part the chromosomal duplication being an effect of colchicine we have been unable to confirm Pelc s unconfirmation Taylor and Woods find no effect of colchicine except some variation in duration of various parts of mitosis but whether labeling was done in the presence of colchicine or not they obtain the same distribution of label They have not been able to show these peculiar results that Pelc claims Does Pelc know that the label is really in DNA or is it just in the nucleus?

L G Lajtha It is in DNA

C P Leblond Dr Hughes you mentioned Dr Koenig s work on the brain. We[4,5] also have seen a certain number of labeled nuclei in the brain using adenine C^{14} and thymidine H^3 We wondered whether it was due to turnover of DNA or synthesis of DNA for formation of new cells (These observations were made in adult rats and mice) Dr Smart noted that a group of such labeled cells was present in one region of the olfactory lobe If there were DNA synthesis with formation of new cells there should be an increase in the amount of DNA in regions such as the olfactory lobe Dr Enesco in our laboratory determined the total DNA content of the olfactory lobe in rats of various ages and found that its DNA content doubled in about thirty days It was concluded that the labeling of cell nuclei in brain after injection of adenine C^{14} or thymidine-H^3 can be explained by DNA synthesis with new formation of cells

W L Hughes Dr Leblond did you see up to 20 to 40 per cent of certain types of cells labeled?

C P Leblond Yes in a subependymal tissue known as the mantle layer The cells of the mantle layer form somewhat less than 1 per cent of the total cells in the olfactory lobe but still in 30 days the total DNA was doubled

W I Hughes It is important to examine critically all the evidence for labeling without synthesis Certainly we don t know all the functions of DNA and it is possible that in certain types of differentiated cells DNA might be operating quite differently than in the liver and other tissues which have been studied most extensively and in which turnover has not been found

L S Kelly The results of Ficq and Pavan[6] in giant salivary gland chromosomes should be mentioned At certain stages of larval development specific chromosome brands increase their DNA content and incorporate thymidine without DNA synthesis in other regions of the chromosome

W L. Hughes I don t object to this I think that there are two points relative to turnover Is it DNA? Is there no extra DNA? These are cases where there is obviously extra DNA

L D Hamilton In fact the amount of DNA labeled in the chromosome is less than five per cent of the total DNA in the chromosome

W L Hughes Is it merely turnover or addition?

L D Hamilton Addition

A Bendich Some years ago we examined the question of whether or not DNA was synthesized in the brain of adult rats We used C^{14} formic acid in metabolic experiments extending over a period of 24 days Although there was extensive incorporation into the RNA of the brain with a rather slow turnover there was no evidence of incorporation of the isotope of the precursor into DNA in the brain[7]

E P Cronkite We observed DNA labeling in the brain of our first patient who had repeated injections of tritiated thymidine Were one estimating DNA synthesis by determining grossly the specific activity of DNA in whole tissue I am quite certain that one would have concluded that there was a certain amount of DNA formed in the brain Looking at the brain cells autoradiographically it is quite evident that all of the DNA is synthesized by the microglial cells and not by the neurons Hence there is a population of cells not connected with the neurons that turns over DNA by cell division

W L Hughes If DNA does not turn over it should prove an excellent system to utilize the hydrogen dating technic Since H^3 has a half life of 12 years the DNA in nonrenewing tissues such as the brains of old people should have much less H^3 than at birth

I Chargaff Is there any evidence that

DNA in *Escherichia coli* exists in the double helical structure?

L D HAMILTON I don t understand to which preparation Dr Chargaff refers The only x ray diffraction photograph of DNA from *E coli* in the literature[8] is the sharp pattern given by the *A* configuration at 75 per cent relative humidity characteristic of the double helical configuration Furthermore some years ago DNA was prepared from *E coli* harvested during their most rapid growth (log phase) and also after most growth had ceased Both gave the same crystalline *A* x ray diffraction patterns This suggests that DNA maintains the same double helical configuration in cells whatever their stage of growth Only a small fraction of the DNA molecules will be engaged in the actual process of duplication and hence we have no information on the structure during the process itself[9]

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Speculations on the Significance of Thymidylic Acid Synthesis in the Regulation of Cellular Proliferation

By Morris Friedkin

WITHIN the polynucleotide polymer that we call DNA is a deoxynucleotide that may play a unique role not shared by the other deoxynucleotides in *the regulation of cellular proliferation. This deoxynucleotide, thymidylic acid,* contains two units of unusual biochemical interest: thymine and deoxyribose. RNA and DNA share adenine, guanine, cytosine and phosphate, but thymine and deoxyribose are components of DNA and not of RNA. A small amount of thymine bound to ribose has been found in certain RNA, but in general thymine bound to deoxyribose is characteristic of DNA.

It is a rather striking fact that no coenzyme containing either thymine or deoxyribose is known. Functions for the various ribonucleotides of RNA are well known, *but as yet no catalytic or coenzymatic function can be attributed to a* deoxynucleotide. Deoxycytidine diphosphocholine and deoxycytidine diphosphoethanolamine have been isolated, but the functions of these compounds are unknown.[4,16]

Does the presence of thymine rather than uracil in DNA have any special biologic significance? Scheme A (fig. 1), a mixture of fact and fiction, is presented as a possible biochemical sequence of reactions tried and discarded in evolution in which DNA containing uracil was synthesized. Perhaps a "missing link" cell which uses Scheme A exists somewhere.

In Scheme A (fig. 1) the hypothetical (likely, but as yet not demonstrated) conversion of ribose to deoxyribose is shown to occur for each of the four nucleotides found in RNA.[10] Each of the four deoxynucleotides is phosphorylated to the corresponding deoxynucleoside triphosphate. The four deoxynucleoside triphosphates then polymerize to form a polynucleotide containing uracil.[1] DNA, however, does not ordinarily contain uracil.

In Scheme B (fig. 1) are briefly sketched biochemical events leading to the *synthesis of DNA as they are now known. The conversion of ribose to deoxyribose for each of the four nucleotides found in RNA is considered a likely but* as yet unproved pathway.[10] The deoxynucleotides of adenine, guanine and cytosine are phosphorylated to the corresponding deoxynucleoside triphosphates,[11] but the phosphorylation of deoxyuridylic acid to the triphosphate does *not* occur.[8] Instead, deoxyuridylic acid must first be methylated, and the thymidylic acid thus formed can be phosphorylated to the triphosphate.[11]

If Scheme A and Scheme B are compared, the methylation of deoxyuridylic acid appears as a shunt pathway. Why did this "methyl shunt" come into existence? After all, uracil and thymine are very similar. Uracil is an acceptable com

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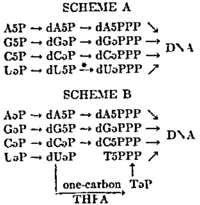

FIG. 1.—Known and hypothetical pathways of DNA synthesis starting with ribonucleotides with special emphasis on the "methyl shunt" concept. The abbreviations used are: 5'-phosphates of adenosine, guanosine, cytidine, uridine (A5P, G5P, C5P, U5P); 5'-phosphates of deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine, thymidine (dA5P, dG5P, dC5P, dU5P, T5P); 5'-triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine, thymidine (dA5PPP, dG5PPP, dC5PPP, dU5PPP, T5PPP); deoxyribonucleic acid (DNA); THFA (tetrahydrofolic acid).

ponent of RNA—why not of DNA? How does the emergence of such a "methyl shunt" in biochemical evolution (if indeed it occurred this way!) provide a cell with more survival value?

The main thesis of this paper is that the "methyl shunt," by operating only a step or two removed from the final polymerization of deoxynucleoside triphosphates into DNA, could provide the cell with a very sensitive means of controlling DNA synthesis.

Kornberg's group has clearly shown that four deoxynucleoside triphosphates must be present for DNA synthesis to occur.[2] If any one of the deoxynucleotides be omitted, DNA synthesis does not occur. The "methyl shunt" appears to be especially sensitive to chemical insult. A block here effectively leads to a halt in DNA synthesis by depriving the DNA polymerase of an essential nucleotide. *Here the earliest inhibitory effects of antifolic agents*[3,7] *and of fluorodeoxyuridylic acid*[4] are manifest. Cohen has made the important observation that *E. coli* cells grown under conditions of thymine deficiency cannot synthesize DNA and that they irreversibly lose the power to proliferate.[5]

A reckless extension of the "methyl shunt" concept is that a bypass would lead to uncontrolled cellular proliferation. For example, all studies of DNA synthesis with labeled thymidine are dependent on the phosphorylation of thymidine first to thymidylic acid and then to thymidine triphosphate. These reactions constitute a bypass of the "methyl shunt." It could be argued that flooding a cell with thymidine would indeed accelerate proliferative processes, but conclusive data for this is lacking.

Several years ago we noted that increasing the level of thymidine C^{14} increases the uptake of the labeled deoxynucleoside into DNA of chick embryos.[6] We concluded that since the endogenous pool of thymidine must be quite low, the increased uptake of labeled thymidine was due to an absolute increase of thymidine incorporation into DNA This could also be interpreted as an absolute increase of DNA synthesis

It must be emphasized that the mere presence of thymidine does not necessarily lead immediately to DNA synthesis If this were true all cells would incorporate labeled thymidine into DNA Actually, only those cells that are already in a state of rapid proliferation incorporate thymidine This is the key to the usefulness of labeled thymidine as an indicator of multiplicative growth[8] These considerations show that the "methyl shunt" concept should not be ridden too hard, all control of proliferation certainly cannot reside here The other deoxynucleoside phosphates must be formed, too, before thymidylic acid can become a limiting or

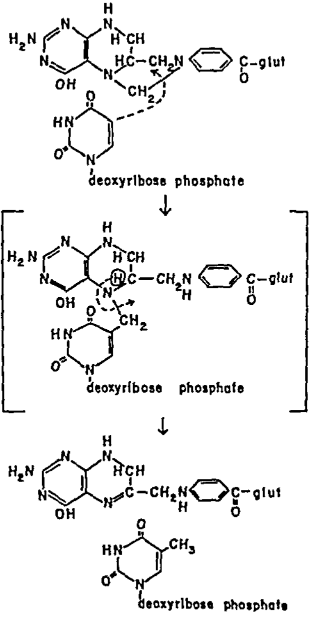

Fig 2 —A speculative mechanism for the formation of thymidylic acid

accelerative factor The DNA already present in a cell conceivably must have a proper or receptive configuration to act as primer for further DNA synthesis catalyzed by 'polymerase'"

We are interested in the mechanism by which the deoxyuridylic acid becomes methylated[8] This is the key reaction of the methyl shunt Folic acid derivatives are definitely implicated It is possible that vitamin B_{12} may also be involved, but there is no conclusive data for this supposition The components of an in vitro system that we have used in our studies are deoxyuridylic acid formaldehyde, tetrahydrofolic acid, magnesium chloride, and an enzyme prepared from extracts of *E coli* Formaldehyde can react enzymatically or nonenzymatically with tetrahydrofolic acid (THFA) to form a cyclic compound in which a methylene group connects the N^5 and N^{10} positions of the reduced pteridine Normally this cyclic compound is generated from serine and THFA These facts have emerged from the research of many laboratories[9]

We have been attempting to elucidate the mechanism of the transfer of the methylene carbon to deoxyuridylic acid and its final reduction to a methyl group—the complete reaction constituting thymidylic acid synthesis O Dell et al[12] suggested in 1947 that tetrahydrofolic acid because of its ease of oxidation may serve as a catalyst in some unknown oxidation reduction system Evidence for a direct transfer of hydrogen from THFA to thymidylic acid has been obtained by incubating tritiated THFA with formaldehyde, deoxyuridylic acid, $MgCl_2$ and *E coli* enzyme[7] The thymidylic acid thus formed was found to be highly radioactive All of the tritium was in thymine none in deoxyribose The results of these tracer studies with tritium support the belief that THFA serves not only as a one-carbon carrier in the enzymatic transfer of a hydroxymethyl group to deoxyuridylic acid but also as a direct hydrogen donor for the reduction of the one-carbon unit to a methyl group How THFA can play this dual role is a matter of speculation

As set forth in figure 2, deoxyuridylic acid cleaves the cyclic pteridine at the

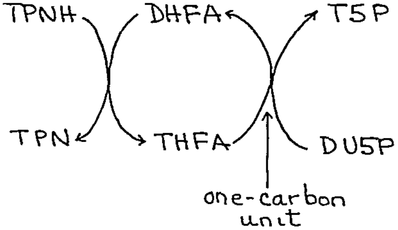

FIG 3 — A biochemical scheme showing how the reduction of dihydrofolic acid (DHFA) by reduced triphosphopyridine nucleotide (TPNH) to tetrahydrofolic acid (THFA) may be coupled to the conversion of deoxyuridylic acid (DU5P) to thymidylic acid (T5P)

bond between the methylene group and N^{10}, thus resulting in the formation of a labile intermediate in which deoxyuridylic acid is bound to THFA by way of a methylene group attached to N^5. An intramolecular oxidation reduction then yields thymidylic acid and dihydrofolic acid. Thus, for every molecule of thymidylic acid synthesized one molecule of THFA is oxidized and must therefore be regenerated before it can be used for the next methylation.

It is the formation of tetrahydrofolic acid from dihydrofolic acid and TPNH that is especially sensitive to the antimetabolic effects of the antifolic agents such as aminopterin.[9] The inhibition of dihydrofolic acid reductase, the enzyme that catalyzes the formation of THFA, should be more critical in a scheme where

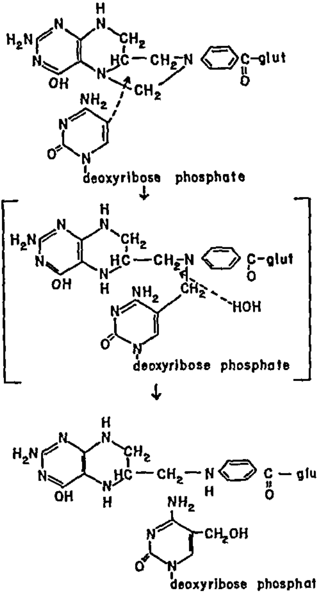

FIG. 4.—A speculative mechanism for the formation of hydroxymethyldeoxycytidylic acid

in THFA acts as a hydrogen donor as well as a one carbon carrier (such as in the synthesis of thymidylic acid) than in a pathway wherein THFA acts only as a one-carbon carrier (such as in the serine glycine interconversion) Perhaps under normal situations the level of TPNH may be an important factor in the control of cellular proliferation by regulating the rate of synthesis of thymidylic acid (fig 3)

In certain phage the cytosine of the phage DNA is replaced by hydroxymethylcytosine It has been shown that the hydroxymethylation of deoxycytidylic acid has requirements very similar to those of the methylation of deoxyuridylic acid formaldehyde serves as the one carbon donor and THFA as a carrier[5] The enzyme for the hydroxymethylation only emerges upon infection of *E coli* by the phage

In the absence of conclusive data, I would like to speculate on how the cyclic pteridine can serve only as a carrier of a hydroxymethyl group to deoxycytidylic acid and not as a hydrogen donor This is in contrast to thymidylic acid synthesis where the cyclic pteridine has a dual role In figure 4 deoxycytidylic acid is shown cleaving the cyclic pteridine at the bond between the methylene group and N^5 thus resulting in the formation of a labile intermediate in which deoxycytidylic acid is bound to THFA by way of a methylene group attached to N^{10} In this case an intramolecular oxidation reduction cannot yield a new methyl group as proposed in figure 2 Hydrolysis would lead to the formation of hydroxymethyl deoxycytidylic acid and THFA

In systems where 5 methyl deoxycytidylic acid is found the sequence of reactions might be analogous to those shown in figure 2 for thymidylic acid synthesis It seems likely but unproven that for the synthesis of methionine from homocysteine *THFA again may serve in the dual role of a one carbon carrier* and a reducing agent

ADDENDUM

In a recent paper (Humphreys G K and Greenberg D M Studies on the conversion of deoxyuridylic acid to thymidylic acid by a soluble extract from rat thymus Arch Biochem & Biophys *78* 275–287 1958) the authors have independently proposed a mechanism for thymidylic acid synthesis which coincides with that formulated in figure 2

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Metabolic Products Formed from Thymidine

By VAN R. POTTER

STUDIES involving the use of labeled thymidine for specifically labeling the DNA of cells for subsequent autoradiography naturally raise questions about other possible fates of the labeled material. The work carried on by my group includes some experiments that may be of peripheral interest, although they have not been carried out with that purpose in mind. Only such data as may bear on the central question will be mentioned.

The metabolism of thymidine (fig. 1) naturally falls into two main categories: (1) conversion to DNA and (2) conversion to breakdown products. More recently a third pathway, too early to classify as belonging to one of the two main categories, has arisen. This is (3) conversion to RNA. The latter pathway is perhaps not established, but the implications are of such obvious interest to the present discussion that the available data will be briefly mentioned.

OCCURENCE OF THYMINE IN RNA

In 1958 Littlefield and Dunn[7] reported the natural occurrence of thymine in RNA from *E. coli* B/r and 15T- and from *Aerobacter Aerogenes*. In all cases the amount was small, equivalent to about 1 molecule per 100 molecules of uracil. Even this small amount was not detectable in rabbit liver or in rat liver microsome fraction. It was shown that the thymine of bacterial RNA was present as a ribo-derivative. Both thymine ribonucleotide and thymine riboside were isolated. More recently Amos and Korn[1] have examined the RNA from *E. coli* K 12 and reported the presence of 5-methylcytidylic acid to the extent of 1 to 2 per cent of the total nucleotide of the RNA. They questioned the findings of Littlefield and Dunn on the basis that the prolonged alkaline hydrolysis was sufficient to hydrolyze 5-methylcytidylic to thymidylic acid (presumably they meant ribothymidylic, since the new nucleotide was a ribotide, not be to confused with 5-methyl deoxycytidylic acid). Their comment is valid but would not apply to certain experiments in which Littlefield and Dunn used snake venom to obtain thymine riboside, although further work with purified enzymatic degradation is needed. At this time the occurrence of ribothymidylic acid free or in RNA must be regarded as tentative and in trace amounts at most. Nevertheless, some support from another direction has come from experiments by Griffin, Todd and Rich,[6] who prepared ribothymidine 5′-diphosphate chemically and showed that it was converted to polyribothymidylic acid by the Ochoa enzyme.

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The facilities for measuring radioactivity are operated under the direction of Dr. Charles Heidelberger. The assistance of Mr. Harry Ludwig in setting up the recording equipment is also gratefully noted.

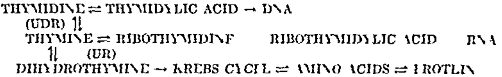
THYMIDINE ⇌ THYMIDYLIC ACID → DNA
(UDR)
THYMINE ⇌ RIBOTHYMIDINE
RIBOTHYMIDYLIC ACID
RNA
(UR)
DIHYDROTHYMINE → KREBS CYCLE ⇌ AMINO ACIDS ⇌ PROTEIN

Fig 1 — Alternative pathways of thymidine metabolism The nomenclature for the riboside derivatives of thymine is not established and the terms used here are those used by Griffin Todd and Rich[6]

Formation of Ribothymidine in Homogenates

Recently Dr deVerdier in my laboratory has obtained evidence for the production of ribothymidine in homogenates of liver and of hepatoma (Dunning LC-18) *These studies probably represent a further exploration of reactions originally* studied by Friedkin and Roberts[4, 5] These workers were concerned with the following reaction

1 *Thymidine + inorganic phosphate ⇌ thymine + deoxyribose 1 phosphate* With a purified enzyme preparation they showed that uracil and thymine could be used interchangeably but when ribose-1 phosphate was substituted the reaction was much less effective and the question of the existence of a separate enzyme for the following reaction was left open

2 Uridine + inorganic phosphate ⇌ uracil + ribose 1 phosphate Their work was based *on reactions* studied *in the presence of an enzyme that was fractionated* to yield reaction No 1 and cannot be expected to answer the question as to the relative proportions of these reactions in the original tissues

Our current studies are based on the use of C^{14} labeled substrates and are still preliminary but some of the findings may be of interest

The following reactions have been studied

3 $(T^* \rightleftharpoons Tdr) \rightarrow (T^* \rightleftharpoons T^*dr)$

4 $(U^* \rightleftharpoons Udr) \rightarrow (U^* \rightleftharpoons U^*dr)$

5 $(T^* + Udr) \rightarrow (T^* + Udr \rightleftharpoons U + T^*dr)$

All of these reactions appear to be catalyzed by a single enzyme which is probably identical to *thymidine phosphorylase of Friedkin and Roberts*[4, 5] *In addition* Dr deVerdier has studied the following reactions

6 $(U^* \rightleftharpoons Ur) \rightarrow (U^* \rightleftharpoons U^*r)$

7 $(T^* + Ur) \rightarrow (T^* + Ur \rightleftharpoons U + T^*r)$

These reactions are probably catalyzed by an enzyme not identical to thymidine phosphorylase but probably the same as the uridine phosphorylase of Cardini et al referred to by Friedkin and Roberts[4] The evidence for the occurrence of reactions 6 and 7 is still not backed up by chemical analyses but rests largely on the following facts (1) rat liver and the Dunning hepatoma differ markedly in the relative amounts of thymidine and uridine phosphorylases (2) the radioactive product in reaction 7 *is not identical to the product formed in reactions 3* or 5 (3) the radioactive product in reaction 7 is formed at the same rate as U^*r

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* Abbreviations T = thymine U = uracil C = cytosine A = adenine Tdr = thymidine Udr = deoxyuridine Cdr = deoxycytidine Adr = deoxyadenosine Ur = uridine Cr = cytidine Ar = adenosine *The asterisk denotes radioactivity*

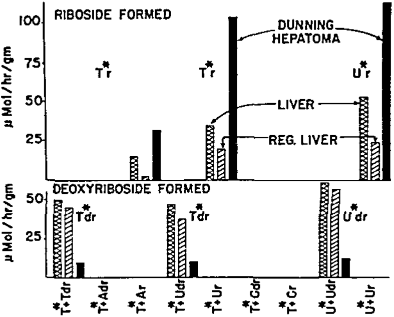

FIG 2 —Isotopic exchange rates between C^{14} labeled thymine (T*) or uracil (U*) and ribonucleosides (r) or deoxyribonucleosides (dr) of thymine (T) adenine (A) uracil (U) or cytosine (C) in the presence of whole homogenates of rat liver regenerating rat liver or Dunning I C 18 hepatoma calculated according to Duffield and Calvin [2] The substrates for nine different reaction mixtures are indicated along the abscissa and the results obtained with the three types of tissue are indicated for each reaction mixture in the appropriate space above the reaction mixture The upper part of the chart shows the amount of ribosides formed (T*r or U*r) and the lower part shows the amount of deoxyribosides formed (T*dr or U*dr) in the same reaction mixture Where no bars are shown the amount of product was too small to be indicated The intent of the figure is to show how the ratios of liver regenerating liver hepatoma for the T*r reaction are similar to the U*r reaction (upper chart) but differ markedly from the T*dr and U*dr ratios shown in the lower chart *The asterisk denotes radioactivity*

in the tissues studied, (4) the radioactive product in reaction 7 is formed only in the presence of Ur and is not formed in the presence of Udr It is tentatively assumed that the radioactive product in reaction 7 is thymine riboside also called ribothymidine This product has previously been found by Fink et al [3] in rat liver slices The remainder of the chart shows that reactions involving adenine or cytosine nucleosides were weak or negligible, as compared with the uracil and thymine nucleosides

Further studies will be needed to characterize chemically the ribothymidine, and to learn whether it can be enzymatically phosphorylated to ribothymidylic acid and whether, under appropriate circumstances, the latter can be incorporated into RNA in mammalian tissues At present the significance of the reaction is completely unknown

The experimental technic will be described in detail elsewhere but here it may be said that protein free samples from reaction mixtures are subjected to paper chromatography, using system No 8 of Fink et al [3] with Whatman No 1 paper The solvent system consists of ethyl acetate, formic acid and water The radioactivity is measured directly with a Nuclear Chicago strip counter and recorded automatically with a Texas Instrument Co Recti-Riter, with concomitant integration and print out reporting by means of a Beckman/Berkeley counter and digital recorder The data are treated according to the kinetic analysis of Duffield and Calvin,[2] which appears to be appropriate for studying reactions of the types shown by equations 3 to 7, and to give deviating results when side reactions supervene The data reported have been based on the use of whole homogenates Additional studies have revealed that the supernatant fraction reveals greater enzyme activity than the whole homogenate as reported by Friedkin and Roberts,[4] and the data appear to be closer to the theoretical model of Duffield and Calvin [2]

CATABOLISM OF THYMIDINE

It has been observed that thymine is a poor precursor of DNA, while thymidine is a good precursor The reason for this must lie in the fact that thymidine is less readily broken down than is thymine Some unpublished studies on the rate of CO_2 output from labeled thymine are shown in figures 3 and 4, in which intact rats were used Carbon 2 of thymine was almost completely lost within two hours, while with thymidine, nearly 40 per cent was retained in the animal—but the loss that did occur was complete in about two hours With methyl labeled thymidine, the retention was over 60 per cent, and the period of loss was during the first two hours Since retention in the form of DNA cannot be any greater for methyl labeled thymidine than for thymidine 2 C^{14}, the difference is likely to be due to a greater retention of the carbon from the methyl group in compounds other than DNA, after the pyrimidine ring has been broken These data are on individual rats, more data are obviously needed The present data are sufficient

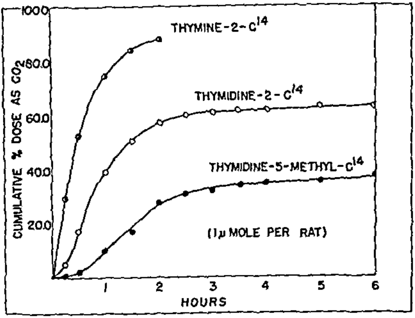

FIG 3 —Conversion of C^{14} labeled thymine and thymidine to $C^{14}O_2$, plotted as cumulative per cent of dose injected versus time

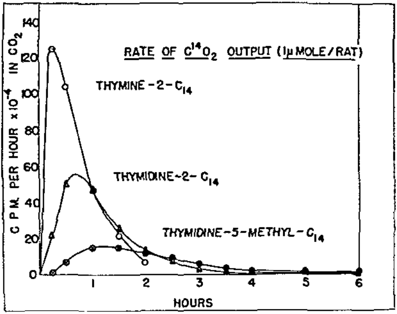

FIG 4 Conversion of C^{14} labeled thymine and thymidine to $C^{14}O_2$ plotted as rate versus time

to show that thymidine is present as such at levels sufficient to be degraded for a very short time and that by the end of two hours an injected dose has been largely converted to other compounds

The nature and magnitude of the pools of compounds formed from thymidine is a matter of some interest to investigators studying cell turnover by means of labeled DNA We have studied the acid soluble fraction from liver regenerating liver and hepatoma tissue 30 minutes after thymidine-H^3 has been injected in vivo A chromatogram from regenerating liver is shown in figure 5 A number of radioactive peaks can be clearly seen These include TMP TDP and TTP whose identification rests largely on the fact that thymidine was injected and because of the work of R L Potter et al [8] who showed that on this chromatographic system certain UV absorbing peaks serve as landmarks for locating the thymidine mono di and tri phosphates In addition certain other radioactive peaks can be seen and some progress has been made in their identification with the help of Miss Anne Brumm and Dr deVerdier The peak near fraction 20 has been called II and contains mainly aspartic acid It may be noted here that glutamic acid is not labeled by thymidine H^3 but is labeled when thymidine C^{14} methyl is given under these conditions We have wondered how the labeling of aspartic by thymidine H^3 could be explained without assuming that it was the H^3 in the methyl group It is of interest that the absence of H^3 from glutamic acid could be readily explained by such an assumption without requiring that C^{14} methyl labeled thymidine should be absent from glutamic It has not been possible to find labeled beta ureido isobutyric acid in peak II possibly earlier time points would reveal radioactivity in this intermediate Peaks IV and V are tentatively identified as succinic and malic acids which would be in line with

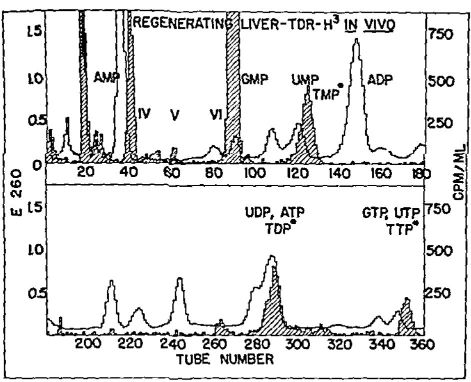

Fig 5 —Chromatogram of the acid-soluble fraction from 24 hour-regenerating rat liver 30 minutes after injection of about 10 μc of thymidine H^3 having a specific activity of 390 μc per μM, or approximately 6 μg total The cross hatched columns represent radioactive fractions with counts per minute (CPM) per ml shown on the right hand ordinate The open columns represent ultraviolet absorbing fractions (wavelength, 260 mμ), as shown on the left hand ordinate Abbreviations represent mono , di , and triphosphates of adenosine, guanosine, uridine (all nonradioactive), and of thymidine (shown with asterisk), plus other radioactive metabolic products of thymidine The radioactive peaks labeled IV, V, and VI are discussed in the text

the labeling of aspartic acid, but again difficult to explain in terms of current assumptions about the labeling of the injected thymidine H^3

We have avoided doing studies with the acid soluble fraction at times other than 30 minutes, until we could identify the peaks already available, since we are interested in the catabolic pathways in relation to the synthetic ones However, our work suggests that much useful information could be gained from a study of the thymidine phosphates alone Such studies have been carried out by R L Potter and colleagues and are probably of greater relevance to the topic under discussion than the data here presented

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Discussion of the Reutilization of DNA and DNA Breakdown Products

L D Hamilton I should like to ask the assembled biochemists and other interested parties their views on the reutilization of DNA and its constituents In the situation in which DNA is labeled (I should mention parenthetically that RNA is usually equally, if not more extensively labeled after most labeled precursors), RNA polynucleotides turn over and cells die perhaps liberating RNA and DNA nucleotides Thus the various labeled constitutents of DNA and RNA are available for reutilization The work of Dancis and Balis[1] suggests that purines liberated into the blood system as a result of nucleic acid catabolism are not reutilized for nucleotide synthesis to any great extent They studied three experimental situations (the pregnant rat, tumor bearing mice, and parabiotic rats) in which an animal with previous labeled nucleic acids was connected by a common circulation to tissue in which the nucleic acids were not labeled, in all three situations they found no significant transfer of label On the other hand, in the tissue culture experiments of Thompson et al,[2] retention of radioactivity in DNA was reduced by one third as a result of adding unlabeled thymidine to the medium This suggested that in their tissue cultures some DNA was broken down and reused locally as deoxynucleosides or deoxynucleotides However, this was only a minor proportion—which indicates that a substantial proportion of DNA must be handed on as a large molecule (polynucleotide) either by cellular division alone or together with or by local reutilization of polynucleotides The transforming activity of bacterial DNA suggests that bacteria can incorporate relatively large portions of the DNA macromolecule into their own pre-existing DNA Fox[3] has shown that the unit absorbed is 1 4× for each effective transformation, i e, 1 4× the DNA content found per cell is incorporated by the population when one cell is transformed This assumes the unit of DNA conferring the new genetic property is present only once in each donor bacterium More recently Fox[4] has been able to show that the size of the piece of DNA that goes into the bacterium transformed is approximately the same as that of the DNA in solution, but direct evidence of the incorporation of the transforming DNA in this situation is still to be obtained

V R Potter It is my opinion that small amounts of thymidine would be reutilized but the tissues of the body would have to battle with the liver because the liver does a pretty good job of converting thymidine to CO_2 In the curves that I presented, we put in 6 μg of thymidine and a very large proportion of this was converted into Krebs cycle intermediates and breakdown products

We have begun a line of experimentation in which various amounts of thymidine are added to liver slices CO$_2$ production and DNA conversion are the two parameters that are measured We have some indication at this moment that the Michaelis constants are different in favor of DNA synthesis at very low levels which was a surprise to us Hence, the question is not whether the contest between liver breaking DNA down and the other tissues reutilizing it can occur, but rather what is the quantification?

D L Kline Do you use regenerating liver?

V R Potter Both regenerating liver and normal liver have the ability to break down thymidine but of course the normal liver has more The experiment that I mentioned was done with slices of regenerating liver

M Friedkin I should like to raise the important question of what happens to DNA when an immature red cell loses its nucleus and becomes a mature red cell As far as I know there are no really good data in this regard It is quite conceivable if this does occur in the bone marrow that the DNA never reaches the liver to be degraded There are probably enough degradative enzymes in bone marrow cells to split the DNA into deoxynucleotides or deoxynucleosides It is quite conceivable that the deoxynucleosides could be phosphorylated and be reutilized by immature marrow cells I think this is an important problem which needs further investigation

A Bendich There are some current experimental data that may give a clue to the extent of possible reutilization of DNA If one administers a precursor such as formate to rats one of the first places where you can pick it up is the small intestine After approximately three weeks you can no longer find label in the intestinal epithelium You do find labeled DNA however, in the liver, spleen kidney pancreas and testes If the DNA of intestinal cells is reutilized, one would expect to find it in the intestine but it seems to go elsewhere One gets a picture that DNA once formed stays for a long time in certain tissues but apparently not in all tissues Certainly there is a tremendous amount of DNA in the intestinal epithelium but once this is labeled it disappears almost quantitatively in the course of three weeks as one would expect in a tissue with rapid renewal but no DNA reutilization

M Friedkin This doesn't really face the problem What we want to know more about is the reutilization of DNA released in the bone marrow when the red cell loses its nucleus

M M Wintrobe It seems to me that Dr Friedkin has asked a very important question which has not yet been answered, namely what happens to the DNA of the nucleated red cell I don't see that Dr Bendich s explanation begins to answer it The disappearance of DNA from the intestinal epithelium could be explained by shedding of intestinal epithelium and its excretion This is a biologic phenomenon, not a chemical one

V R Potter The question Dr Friedkin raised is most appropriate Does some DNA break down to nucleotides in a segregated compartment in which the nucleotides are reutilized without ever being dephosphorylated and escaping into the circulation as thymidine? He agrees with me that if it gets into the circulation as thymidine, the liver will degrade a large portion of it I think that one way in which this can be studied is to examine for the presence of deoxyribosidic phosphorylase and various other enzymes involved in the degradation of DNA—enzymes such as those we reported at this meeting

M Friedkin Is it possible that after the red cell loses its nucleus, and therefore its DNA that the white cells can utilize the DNA as a unit? Perhaps the white cell more completely utilizes these nucleotides than would be the case if the deoxynucleotides spilled out into the blood stream There should be a way of testing whether white cells can engulf large molecules such as DNA If they were labeled, you might see it by autoradiographic technics Has this been done by anyone?

L S Kelly One must distinguish between the possible reutilization of large chunks of DNA and the reutilization of the constituent bases derived from the breakdown of RNA and DNA It seems reasonable to assume that some of the nucleic acid breakdown products re-enter the acid soluble pool and are reutilized for nucleic acid synthesis in addition to the bases derived from *de novo* synthesis However, at present the fraction of bases derived from each source is not known and it could well differ for different tissues

L. G Lajtha We have calculated from the number of cells produced that about 2.8 grams of purines are produced per day in a fully grown human If this is all neosynthesis, then a very large amount of uric acid ought to be excreted On the other hand the daily uric acid excretion is less than half a gram We do know that there is some neosynthesis in the liver We do know that we take in diet about a quarter of a gram of purines which on the face of it means that at least 80 per cent of the daily purine requirement is reutilization with possibly 10 to 20 per cent neosynthesis The vast majority is certainly reutilized

E Chargaff Well isn t it possible that you have reutilization at different levels? You have so many different enzymes to take care of

L G Lajtha I don t know at what level they are reutilized

E Chargaff They are probably reutilized in different tissues at different levels

L G Lajtha At any time in bone marrow a certain proportion of the cells will be in the process of DNA synthesis *and will take up a label When those cells* extrude their nuclei and DNA is liberated in circulation it will be diluted over a longer period than the actual initial label was available in circulation Moreover all the nuclei won t be released synchronously

N B Kurnick There is some clinical evidence that at least a good portion of the DNA derived from the extrusion of erythroblastic nuclei is not reutilized but is excreted as uric acid In polycythemia vera there is a great increase in the excretion of uric acid which gives rise to hyperuricemia

J Ambrus The mature red cell not only lacks a nucleus but it also lacks lysosomes yet early stem cells show morphologic structures which could be interpreted as being lysosomes When the red cell extrudes the nucleus it might also extrude the lysosomes These are known to contain enzymes which normally degrade DNA The products of the nucleus might be reutilized then for erythropoiesis in the same location

A Bendich Beta aminoisobutyric acid which is excreted in the urine is a specific end product of thymidine catabolism Dr Potter just told me that about 100 to 200 mg was excreted daily in the urine If it turns out that this is the situation then a considerable portion of the thymidine in the body is no longer available for resynthesis of DNA

F P Cronkite After an injection of tritiated thymidine into a human approximately 25 to 50 per cent is recoverable in the form of tritiated water Another 5 to 10 per cent is recoverable during the first few days in the form of beta aminoisobutyric acid and other nonvolatile tritium compounds in the urine

A large number of normoblastic nuclei are extruded each day, about 2×10^{11} These are difficult to see in the marrow The nuclei are extruded extravascularly and it is not until after the nuclei have been extruded that the red cells get out into the circulation Therefore it seems logical to assume that these nuclei are broken down extravascularly very rapidly degraded and if they go down to the various purine and pyrimidine bases then I suspect about the same percentage of the thymine as Dr Bendich has mentioned would go to the beta aminoisobutyric acid and tritiated *water*

J Davidson There is efficient reutilization of Fe^{59} from hemoglobin following the death of the red cell (RBC) If you follow the disappearance curve of RBC Fe^{59} you fail to see a good inflection point at the time that labeled cells die The curve has a long tail due to reutilization of the iron When granulocytes are labeled with C^{14} purines the shape of the disappearance curve and the absence of a tail would in itself seem to be evidence of poor reutilization of the labeled purines

E F Osgood Dr Friedkin I can provide a tool by which you can get a clearcut answer on this question We now have long term cultures of all varieties of hemic cells * If you cultured the erythrocytic series added tritium labeled thymidine washed the culture thoroughly and then added a culture of an entirely different cell type you could see if the tritium labeled DNA was utilized by these cells of the second type following extrusion of the erythrocytic nucleus

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W L Hughes There are some data bearing on the subject of reutilization that bother me *If one injects thymidine into an animal or human and studies the formation of tritium water* (HTO) it rises in the course of perhaps one or two hours to a maximum then stays almost constant and then slowly disappears over a long time because water turnover is slow When plotted exponentially this component appears to be a straight line This is our mouse data If this is true there doesn t seem to be any room for the formation of tritium water from the degradation of DNA The question arises how sensitive is the technic?

V R Potter What is being plotted?

W L Hughes *HTO concentration in the animal as a whole as a function of time As I said following injection of H³ thymidine it rises rapidly to a maximum This is the labeling time At this time we think all the tritium of the injected thymidine is either water or DNA From this time on HTO formation stops and therefore its concentration slowly falls* We get exactly the same curve if we inject HTO instead of tritiated thymidine

If the fraction of tritium incorporated into red cell DNA represents a large portion of the total uptake of DNA—I don t know whether this is true—and if when they lose *their nuclei degradation products as small as thymidine are formed* then we ought to get some sort of a bump in the curve at that time We haven t seen this The question then is whether or not the percentage of the total H³ in the nuclei of cells with a short nuclear life is great enough to produce an increment of tritiated water big enough for us to detect

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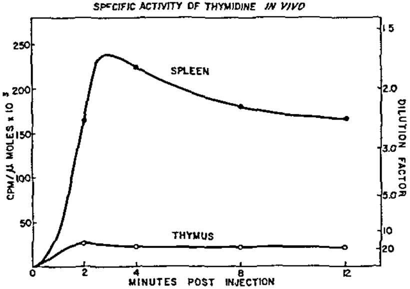
4 —— *Personal communication to L D* Hamilton

Discussion of Availability Time of Labeled DNA Precursors

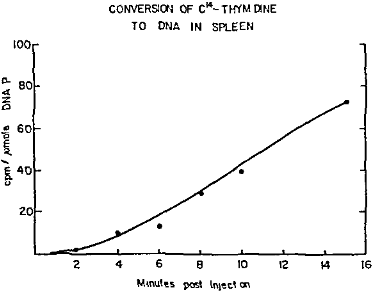
C P Leblond Would Dr Potter give us some information about the specific activity of thymidine?

R L Potter Following a single intraperitoneal injection of C^{14} thymidine into a rat that weighs 180 grams the specific activity of this compound in the thymus reaches a maximum in *two minutes* and in the spleen in somewhere between two and four minutes (Discussion fig 1) In collaboration with my colleague Dr Nygaard it was possible to show that the conversion of C^{14} thymidine to DNA in spleen is detectable in two minutes and is virtually a linear function of time for the remainder of the 15 minute period (*Discussion fig* 2)

The third chart (Discussion fig 3) shows the *total* radioactivity of thymidine (TDR) and its nucleotides in the spleen after an injection of 10 micromoles of thymidine in vivo *Note that all three of the nucleotides are labeled at two minutes and that the total activity of the thymidine monophosphate* (TMP) is somewhat higher than thymidine diphosphate (TDP) or thymidine triphosphate (TTP) Note also that the total radioactivity of the thymidine fraction is roughly 100 times that of its nucleotides This suggests that the conversion of thymidine to TMP is a rate *limiting reaction and that the interconversion* of the thymidine nucleotides among



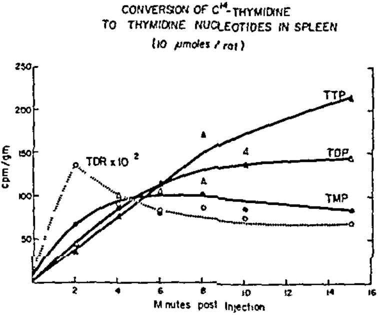
Discussion fig 1 —The specific activity of thymidine following a single intraperitoneal injection The dilution factor (right hand scale) is calculated on the basis of 400 000 CMP/μmole for the injected thymidine



Discussion fig 2

themselves and to DNA is quite rapid Unfortunately, the extremely small quantity of thymidine nucleotides in spleen (and all other tissues except thymus) almost precludes a determination of specific activity which would allow us to draw definite conclusions regarding pathways

Discussion table 1 gives the *specific activity of thymidine and its nucleotides from* rat thymus Note the sharp drop in specific activity from thymidine to TMP as might have been anticipated from the data in Discussion figure 3 Note also that a further five fold dilution occurs between



Discussion Fig. 3.—The conversion of C^{14} thymidine (TDR) to thymidine monophosphate (TMP), thymidine diphosphate (TDP) and thymidine triphosphate (TTP) *in the spleen of the rat following an injection of 10 μmoles (3μc) of C^{14} thymidine* cpm/gm refers to counts per minute per gram of splenic tissue

Discussion Table 1.—*The Specific Activity of Thymidine and Thymidine Nucleotides in Thymus in Vivo*

Compound	Minutes Post-injection		
	4	8	12
Thymidine	22,400	21,000	21,700
Thymidylic acid	743	1,100	1,742
Thymidine diphosphate	126	213	396
Thymidine triphosphate	131	233	315

TMP and either TDP or TTP. Since these last two compounds have the same specific activity, it is impossible to determine which is precursor and which is product. It therefore appears that two rate limiting reactions occur in the nucleotide series TDR → TMP and TMP → TTP (or TDP whichever appears first).

D. Nathan: Would Dr. Hughes comment on the possibility of continued labeling with tritiated thymidine in the marrow? Glycine-2-C^{14} labels red cells continuously for as long as 80 days after its administration, as shown by Berlin et al.[1]

W. L. Hughes: We found that when we rapidly injected thymidine into a mouse, a large percentage of it, 50 per cent or more, was converted to tritiated water, and the rest could not be found. Presumably if we broke down DNA we could find the rest of it. We have not looked for it. In the animal as a whole, tritiated water formation stops about an hour or two after injection. How long does thymidine remain in a synthesizing cell? It seems to me quite possible that a cell which is synthesizing DNA might lack degradative enzymes purposely, and therefore the labeling period might be somewhat longer—I can't believe days, but certainly hours rather than minutes. A labeling period of 80 days must result from reutilization of label.

E. Chargaff: This may be true, but for *E. coli* you wouldn't maintain that. There seems to be another form of compartmentalization rather than just different cells.

might be a difference in the enzyme content or activity in these cells. I'm not sure that your point would actually hold

E. Chargaff: I think it does

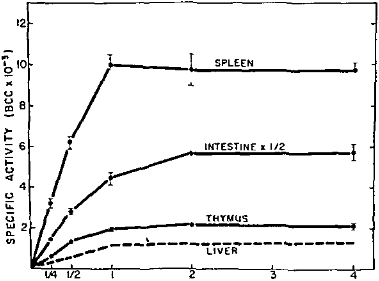
G. J. Friou: It seems possible that cells that are in the process of growth and not near the time for division might be able to break down thymidine whereas those that are multiplying might lose this capacity or it might be inhibited

E. Chargaff: The chemist goes by what he can isolate. It is hard to speculate about what might and might not happen in the growing cell. We do know that enzymes of degradation and synthesis can occur together in the same cell. It is just another version of the simple statement that we have not the slightest idea of what we mean by *the organization of the cell*, and I believe this applies even to some of the rest of the discussion. Obviously there is a difference between a growing and a resting culture, but we do know that in cases of partially stopped growth, as for instance in the presence of chloroamphenicol, there can be *synthesis and breakdown of RNA at the same time*

C. P. Leblond: When a cell dies the speed with which the DNA is eliminated is amazing. If you give colchicine you may find in the epidermis three per cent of the cells in colchicine-blocked metaphase six to eight hours after administration. By 12 to 14 hours these cells are gone. This observation shows how rapidly the body gets rid of cells once they are dead. In contrast, as *long as cells are alive they tend to persist indefinitely. If you inject thymidine-H^3 into* 3 day old rats and sacrifice them at intervals up to six months after injection you find that in some tissues the radioautographic reaction overlying the nuclei may be just as intense at six months as at three days after injection.[2] This result *indicates complete stability of DNA as long* as the cell is alive, in contrast with the speed with which the dead cells vanishes

V. R. Potter: If you plot the total acid soluble radioactivity in the blood after an injection of labeled thymidine it drops to zero at the end of two hours. This shows *that there is no circulating radioactivity* derived from thymidine either as thymidine or any other catabolic product. This coincides precisely with our curves on CO output. This means that as soon as the radioactivity gets into the blood it is cleared by the liver. The crucial experi*ment is simply to examine the acid soluble* extract of the bone marrow, the thymus or any other suspected compartment to find out if radioactivity is being segregated there in smaller acid soluble fragments since you know that if it gets into the blood stream it will be cleared

E. P. Cronkite: *The problem of avail*



Discussion fig 4

ability of thymidine for synthesis of new DNA has concerned us. It is essential that the *effective availability time be a short* fraction of total DNA synthesis time in order to get a clear-cut interpretation of the ultimate kinetics of cell division. The rather simple experiment was done of putting a small blood pressure cuff around the abdomen of a guinea pig. Inflation to 300 mm Hg occluded the circulation below the blood pressure cuff. Then thymidine was injected into the jugular vein and the tourniquets released at various intervals. An hour thereafter the animals were killed and autoradiographs of bone marrow and lymph nodes of the upper extremity were compared with those of the lower extremity. Fifteen minutes of occlusion produced substantial reduction in the grain count in the marrow of the lower extremities. However, after 40 minutes of occlusion there was still some labeling in the lower extremities, the ratio of labeling between upper and lower extremities being about 9:1. We have not studied longer time intervals nor do I know whether a guinea pig would tolerate occlusion of the circulation for much longer periods, but certainly there is some form in which the thymidine or some product is available after 40 minutes and which can be *transported from the* upper part of the body to the marrow of the lower extremities.

R. L. POTTER: The specific activity of DNA *was determined in four different tissues* following a single dose of 3 μc of C^{14} thymidine intraperitoneally.[3] Labeling in the spleen, thymus, and liver was almost *linear for the first hour with little or no labeling in the second hour. In the intestine* there may be some labeling in the second hour. I think it is quite clear that following a single dose of thymidine, labeling of DNA is essentially complete within one hour in most tissues.

G. BRECHER: *Dr Potter has indicated* that thymidine incorporation into DNA goes on for an hour after a single injection. Could he elaborate on what happens in this hour? *Is the thymidine available for an* hour? Is it stored in the cell? As soon as it gets into the cell is it immediately incorporated in the DNA? I think some of the discussions on the meaning of the grain counts of individual cells, whether they really indicate the speed of DNA synthesis and thereby indirectly the direction of DNA synthesis in a single cell, will depend on this *type of consideration*.

R. L. POTTER: It is clear from the previous slides that the radioactivity of the thymidine triphosphate increases quite rapidly. *From the fall in specific activity* of thymidine as well as the fall in *total soluble radioactivity* I would guess the specific activity of TTP would continue to increase *for 20 to 30 minutes*. By this time the specific activity of thymidine will be decreasing and so will that of thymidylic acid. Whether or not any of these curves cross over one another, they will all be back to a very low specific activity by the end of the first hour. A very important question which we have not yet resolved is *whether all of the cells in a tissue convert* thymidine to its nucleotides or only those actually engaged in DNA synthesis.

M. FRIEDKIN: The enzymes involved in DNA synthesis are more active in those cells that are rapidly dividing than in those that are resting. The activity of enzymes involved in DNA synthesis appears to be *inversely proportional to the generation* time of cell. *E. coli* cells (generation time 0.5 hours) contain approximately 40 times as much polymerase (the enzyme involved *in DNA synthesis*) *as is present in bone* marrow cells (generation time 24 hours). The activity of the methylating enzyme involved in thymidylic acid synthesis is *much higher in more rapidly dividing cells* such as *E. coli*, than in bone marrow.

I think Dr Potter has evidence that in regenerating liver, the enzymes involved in the phosphorylation of thymidine to *thymidylic acid and to thymidine triphos*phate increase markedly before DNA synthesis starts. *It is very important to de*termine the factors which control the emergence of these enzymes involved in the synthesis of DNA. Are they always present *and inhibited or do they arise due to* influences that we don't understand?

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Observations on DNA Synthesis by Bone Marrow In Vitro: Stability and Pitfalls

By E. Donnall Thomas

My REMARKS are primarily concerned with observations of DNA synthesis by bone marrow in vitro. The bone marrows in our experiments were either human marrow or rabbit marrow. No significant differences were noted. There is little cell division during our short term cultures. Hence, we must regard this DNA synthesis as "intent to divide" and therefore a manifestation of potential ability to proliferate.

DNA synthesis by marrow in vitro requires intact cells. Quick freezing prior to incubation results in no detectable synthesis. The supernatant medium at the end of incubation shows no activity in the DNA fraction, indicating that there is no significant breakdown of labeled cells in periods up to 24 hours.

DNA synthesis has been measured by two methods: (1) Incorporation of C^{14} formate into DNA thymine[1]; (2) incorporation of H^3 thymidine into DNA thymine. These chemical methods indicate over-all DNA synthesis and do not indicate the relative contribution by any particular cell type.

The stability of DNA has been evaluated in two experiments. In the first experiment (table 1), marrow was incubated in C^{14} formate for two or four hours. The cells were then washed and incubated for an additional two or four hours in unlabeled formate. The specific activity of the DNA thymine increased during the incubation with unlabeled formate, indicating continued synthesis of DNA from labeled compounds already in the cell. No evidence of exchange of unlabeled formate with C^{14} in DNA could be detected.

In the second experiment (table 2), H^3 thymidine was injected intravenously into each of two rabbits. They were sacrificed at the times indicated, the marrow was removed and incubated in vitro for a period of 24 hours with and without the addition of unlabeled thymidine. There was no decrease in specific activity of the DNA thymidine, indicating its stability under these conditions.

When bone marrow is incubated in vitro with H^3 thymidine, we have observed that the specific activity of the thymine isolated from DNA is about 1/70 that of the H^3 thymidine in the medium. This means that the total amount of DNA synthesized during the period of incubation is quite small. Hence, in the experiment shown in table 2, the DNA synthesis occurring during the incubation with unlabeled thymidine is not large enough to cause a detectable decrease in specific activity.

There are several pitfalls in the measurement of DNA synthesis by marrow in vitro. This process is quite sensitive to changes in pH, with the optimal pH *being about 8.0. Different samples of serum from hematologically normal donors* differ by as much as plus or minus 50 per cent, with regard to their ability to promote DNA synthesis. This interesting property of serum deserves further study. It complicates attempts to assay serum for erythropoietin or for other

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TABLE 1.—*Experimental Conditions. 95 × 10^6 Nucleated Cells per Aliquot*

Aliquot	Incubation in C^{14}-Formate (hours)	Additional Incubation in Nonlabeled Formate (hours)	CPM/μM of Thymine	Average
1	0–2	None	8,640	
2	0–2	None	9,640	9,140
3	0–2	2–4	10,800	
4	0–2	2–4	11,290	11,045
5	0–4	None	13,290	
6	0–4	None	15,190	14,240
7	0–4	4–8	16,950	
8	0–4	4–8	17,750	17,350
9	0–8	None	19,050	
10	0–8	None	19,500	19,275

From Thomas, E. D. and Loehte, H. L., Jr. Desoxyribonucleic acid synthesis by bone marrow cells in vitro. Blood *12:* 1086–1095, 1957.

TABLE 2

Flask	Hours After Injection*	Hours in Vitro	DNA Thymine Specific Activity (CPM/μM)
1	22	0	592
		4	625
		9	626
No Carrier		22	600
		29	726
2	22	0	620
		4	655
		9	604
With Carrier†		22	555
		29	616
3	1.5	0	995
		4	1186
		9	1143
No Carrier		21	1227
		29	1107
4	1.5	0	1086
		4	1125
		9	1043
With Carrier†		21	943
		29	973

* 200 μc. of H^3 thymidine per Kg. body weight injected intravenously.
† 0.1 μM per ml. of medium.

stimulatory or inhibitory agents. A major pitfall is the rapid loss of ability to synthesize DNA by marrow cells kept in vitro. Figure 1 shows this loss in a typical vaccine vial liquid culture.[2] Using several other culture technics, we have not been able to achieve better preservation of DNA synthesis. From this we

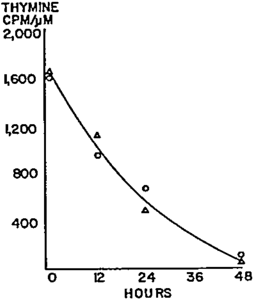

Fig 1 —The effect of the time of preincubation on DNA synthesis by normal bone marrow in vaccine vial culture After the period of culture shown C^{14} formate was added to the vial Incubation was then carried on for an additional 4 hours In the experiment shown the triangles represent a nucleated cell concentration of 3 338 per cu mm and the open circles represent a concentration of 1 730 per cu mm (From Thomas E D and Lochte H L Jr Studies on the biochemical defect of pernicious anemia I In vitro observations on oxygen consumption heme synthesis and deoxyribonucleic acid synthesis by pernicious anemia bone marrow J Clin Invest 37 170 1958)

conclude that great caution should be used in interpreting the results of experiments involving DNA synthesis by marrow in vitro, when the experiments extend beyond a few hours

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Discussion of DNA Synthesis in Bone Marrow Cultures

E E Osgood Dr Thomas what was the concentration of serum used in your cultures? Was it fresh serum or old serum? We have done experiments in long term cultures which suggest that fresh serum contains inhibitors of cell division We set up a series of identical cultures with the exception of varying the amount of serum and comparing old and fresh serum, concentrations of 80 40 and 10 per cent were used, Eagle s medium made up the balance of the solution The growth was identical in each concentration of old serum Growth increased progressively as you reduced the amount of fresh serum and there was far less growth even in the best culture with only 10 per cent fresh serum than in any of those in which old serum was used

E D Thomas Dr Osgood what do you mean by old serum ?

E E Osgood Commercially available Courtland* serum kept in an ordinary refrigerator

E D Thomas With regard to the question about concentration of serum we have used concentrations ranging from 5 to 80 per cent customarily somewhere around 30 to 40 per cent Previously we published a curve showing the effect of the concentration of serum on DNA synthesis[1] No serum at all is certainly bad Five per cent serum will increase the rate of DNA synthesis As I remember if we arbitrarily start the amount of synthesis observed with 80 per cent serum as 100 per cent then no serum is of the order of 15 5 per cent serum brings that up to 25 per cent or so and the curve levels off at about 50 per cent serum We have not found any evidence of toxicity of high concentrations of serum as has been suggested The serum that we used was collected in a batch and stored usually in the deep freeze so by your definition this would be fresh serum

E L Alpen I gather Dr Thomas is reporting specific activity measurements made on cultures at various times without regard to the cellular composition of the system with which he is working It is very important to realize that none of these bone marrow cultures is self sustaining At least I know of no one who has set up a culture where he has the same cellular distribution at 48 hours that he started with After 48 hours of culture with a culture bottle technic using human bone marrow, you have predominantly very late cells which for the most part are mature non DNA synthesizing cells If you look at these cultures autoradiographically, those cells young enough to synthesize DNA are still synthesizing it at the same rate as at the start of the procedure I wonder if Dr Thomas is willing to concede that this might be the reason why 48 hours after a starting culture the DNA incorporation is at a low level

E D Thomas Yes entirely If you keep bone marrow in vitro for any length of time the cells that are going to divide are gone Dr Lajtha has shown this by morphologic technics a long time ago If you are trying to measure DNA synthesis then you have a poor cell population to work with

E L Alpen Does not that mean that you have to study this cell population autoradiographically so that you can look at those cells capable of DNA synthesis?

E D Thomas That's quite right but you are looking at a sample of a cell population that is certainly not representative of the normal sample

L G Lajtha I think there is no disagreement It is a question of definition When one speaks of DNA synthesis one ought to define exactly what one means and when one speaks of the process of DNA synthesis in any one cell then apparently up to the first 48 hours there is no significant change in what happens to any one cell Of course I quite agree with Dr Thomas that the number of cells capable of division decreases very greatly

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Studies on Purine Nucleotide Biosynthesis in the Rabbit Erythrocyte

By Bertram A Lowy

THE PROBLEM of deoxyribonucleic acid formation has many facets, some of which have been discussed earlier In terms of our understanding of the over all biosynthetic mechanism, many uncertainties remain It is generally assumed, for example, that the basic process of deoxyribonucleotide formation occurs at the nucleotide level by reduction of the ribose portion of a ribonucleotide to form the corresponding deoxyribose derivative It may be further assumed therefore that the biosynthetic mechanisms leading to purine and pyrimidine ribonucleotide synthesis are common to both types of nucleic acid, and to the nucleotide coenzymes

In recent years much has been learned about the sequence of events leading to the biosynthesis of the nucleotides There appear to be two general routes leading to purine nucleotide formation In both routes the phosphorylated ribose derivative, 5 phosphoribosylpyrophosphate (PRPP), is involved In the so-called *de novo* pathway, the purine is built up stepwise on the first carbon of the ribose via a number of intermediate reactions in which the amino acids glycine, aspartic acid and glutamine as well as other relatively simple, commonly occurring molecules such as formate and carbon dioxide serve as precursors for specific positions in the purine ring This pathway is summarized very briefly in figure 1 The elegant studies of Buchanan [4] of Greenberg [9] and of others have clarified the individual reactions involved in this biosynthetic mechanism leading to purine nucleotides *de novo* Intermediates have been isolated and their structures have been determined, cofactor requirements have been established and for some reactions purified enzymes have been prepared Since the several precursors of the purine nucleotides are readily present in most cells, major emphasis has been placed upon *de novo* synthesis as the mechanism of purine nucleotide biosynthesis

In the so-called salvage pathway (fig 2) preformed purines, presumably derived from the degradation of nucleic acids nucleotides, or nucleotide coenzymes react with PRPP to form the corresponding nucleotides directly [13] Since purines are negligible dietary constituents it must be assumed that they arise from nucleotides that are synthesized within the organism by the *de novo* pathway, the only known route leading to purine ring formation Except for uric acid, which is not known to participate in nucleotide formation there is a paucity of free purines in the higher forms of life It has been concluded therefore, that free purines originate from nucleotides via the *de novo* pathway and that the salvage pathway, representing a limited means of reutilization is of minor importance

In addition to the utilization of free purines nucleotide formation has also been demonstrated by phosphorylation of nucleosides However this type of reaction appears to be very limited in nature Kornberg and Pricer[17] have de

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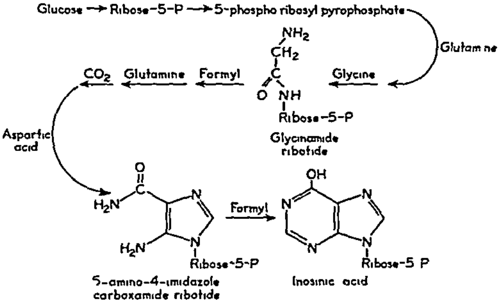

FIG 1—Summary of purine nucleotide biosynthesis by the *de novo* pathway

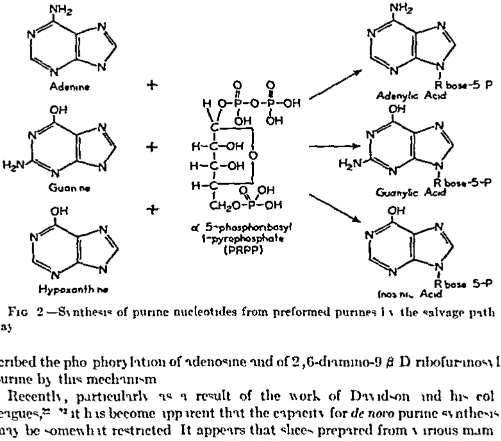

FIG 2—Synthesis of purine nucleotides from preformed purines by the salvage pathway

scribed the phosphorylation of adenosine and of 2,6-diamino-9 β D ribofuranosyl purine by this mechanism

Recently, particularly as a result of the work of Davidson and his colleagues,[22, 23] it has become apparent that the capacity for *de novo* purine synthesis may be somewhat restricted It appears that slices prepared from various mammalian and avian tissues are incapable of *de novo* synthesis in vitro although nucleic acids and acid soluble nucleotides seem to be synthesized in these tissues in vivo The results of these studies have suggested that the ability of the liver to carry out *de novo* purine synthesis to comparable extents under both in vivo and in vitro conditions may be unique. Furthermore, it has been shown that liver extracts, even after boiling, will markedly enhance the in vitro *de novo* synthesis of purines in a number of tissues that are incapable of purine synthesis in the absence of this supplementation. The suggestion has been made that the liver may play a special role in purine nucleotide synthesis. Perhaps it provides to other tissues a necessary cofactor or, more probably, a preformed purine or a partially formed purine precursor which the deficient tissue may then utilize for nucleotide formation. It is rather intriguing to consider the possibility that *de novo* purine synthesis is restricted to a limited number of organs which serve to supply more complex precursors for purine synthesis to tissues that lack the capacity to synthesize them. Pertinent to this possibility is the finding that many tissues with limited or no ability for *de novo* synthesis can utilize preformed purines extensively for purine nucleotide synthesis in vitro.[7, 14, 21] Further suggestive evidence for a special role for the liver has recently been provided by Lajtha and Vane[14] who showed that following hepatectomy there was a marked decrease in the utilization of formate C^{14} for synthesis of the purines of DNA in rabbit bone marrow in vivo, although DNA thymine synthesis proceeded normally. They have suggested that the liver supplies preformed purines to the bone marrow cell, and perhaps to cells of other tissues as well.

Our current studies are concerned with metabolic processes related to the ageing of the erythrocyte, a problem that may appear rather remote from the subject of DNA biochemistry, especially since the mature mammalian erythrocyte represents one of the very few cell types devoid of any form of nucleic acid metabolism. However, our studies are directed toward an understanding of the possible relationship between nucleotide metabolism and viability of this cell. By administration of glycine-2-C^{14}—a precursor of both the heme portion of hemoglobin and of the adenine portion of ATP—to a group of adult rabbits, it has been possible to compare the metabolic stability of these compounds.[17] Unlike the hemoglobin of the red blood cell, which is metabolically stable during the life of the cell in the peripheral circulation,[19] the adenine portion of the ATP of the erythrocyte does not exhibit this stability. One explanation for this finding would be based on a metabolic renewal of the adenine nucleotides during the life span of the cell. This possibility has been examined in vitro by incubating rabbit erythrocytes with radiocarbon labeled glycine or sodium formate, known purine precursors, together with or without the other purine precursors, under a variety of conditions.[18] These experiments resulted in a failure to demonstrate *de novo* synthesis of purine nucleotides, which indicates that the rabbit erythrocyte may not possess the capacity for over-all *de novo* purine synthesis in vitro.

Since the mammalian erythrocyte does not synthesize nucleic acids or proteins, the failure to demonstrate *de novo* purine synthesis was not surprising. However, it appeared possible that, although the mechanism for over-all *de novo* purine synthesis was lacking, the cell might be able to utilize a partially formed purine precursor with conversion to a purine nucleotide, i.e., it might possess part of the over-all biosynthetic mechanism. The compound chosen for study was 5-amino-4-imidazolecarboxamide riboside (fig. 3). This compound, which lacks only a single carbon to complete the purine moiety, is closely related to the corresponding ribotide, the known immediate purine precursor. The incorporation of the missing carbon atom to complete the purine ring takes place via a folic acid dependent formylation.[9]

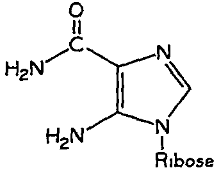

FIG 3 —Structure of 5 amino-4 imidazolecarboxamide riboside

When rabbit erythrocytes were incubated with sodium formate C^{14}, glucose, inosine, and the small molecule purine precursors, no radiocarbon label was detectable in the adenine of the isolated erythrocyte ATP The inosine was added to provide a source of phosphorylated ribose formed by the purine nucleoside phosphorylase reaction When the incubation mixture contained sodium formate C^{14}, aspartic acid glucose, and 5 amino-4 imidazolecarboxamide riboside considerable incorporation of radiocarbon appeared in the adenine portion of the ATP (table 1) The addition of aspartic acid, to provide the source of the 6 amino group, was found to be unnecessary Similar results were also obtained in later experiments in the absence of glucose and when isotonic phosphate buffer, pH 7 2, replaced plasma as the incubation medium

TABLE 1 —*The Biosynthesis of the Adenine of ATP in the Rabbit Erythrocyte in Vitro*

Incubated*		Isolated
Compounds	Concentration	Adenine of ATP
	mmole/hr /100 ml. cell	cpm/µmole
(a) Sodium formate C^{14} (40 µc)	0 2	4
glucose L aspartic acid L glutamine	0 2	
glycine sodium carbonate, inosine	0 1	
(b) Sodium formate C^{14} (40 µc),	0 1	6500
L aspartic acid 5 amino-4 imidazolecarboxamide riboside	0 1	
Glucose	0 2	

* Rabbit erythrocytes (33 ml) incubated in plasma (33 ml) at 37°C for 3 hours

Degradation of the adenine obtained from ATP in a similar experiment indicated that the radioactivity was localized in carbon 2, as would be expected if the radio formate were utilized for completion of the purine ring of the 5 amino 4 imidazolecarboxamide riboside Evidence is provided, therefore, for the existence, within the rabbit erythrocyte of the enzymatic apparatus for the final steps of purine biosynthesis, despite the apparent lack of the over all biosynthetic mechanism One wonders whether this capacity of the erythrocyte represents a functional pathway, necessary for the survival of the cell, or whether it is a non functional remnant from the pre-erythrocyte stages in the bone marrow, when the cell had, perhaps, a limited ability for *de novo* synthesis One wonders also whether a compound such as 5-amino-4 imidazolecarboxamide riboside may be synthesized by the liver for transport to bone marrow or to other tissues for con version to purine nucleotides, in order to satisfy a requirement of that tissue as a result of an inability for over all *de novo* synthesis.

The addition of 5-amino-4-imidazolecarboxamide riboside or of glucose to mouse Ehrlich ascites tumor cells has been found by Thomson, Smellie, and Davidson[13] and by Harrington,[10] to result in a marked enhancement of formate C^{14} incorporation into the purines of both RNA and DNA and into the purines of the acid soluble nucleotides. The function of the riboside was shown to be primarily as a source of ribose. However, when rabbit bone marrow was studied in a similar experiment, enhancement of formate C^{14} incorporation by 5-amino-4-imidazolecarboxamide riboside was also found, although this effect could not be produced when glucose replaced the riboside. These authors conclude that the rate of purine synthesis in the Ehrlich ascites tumor, but not in the rabbit bone marrow, is probably related, to a considerable extent, to the availability of glucose. In the rabbit erythrocyte, our experiments have also indicated that the enhancing effect of 5-amino-4-imidazolecarboxamide riboside cannot be obtained when glucose replaces the riboside.

In order to determine whether the purine guanine could also arise in a manner analogous to that of adenine, methods to be reported elsewhere were developed for the isolation of guanosine triphosphate (GTP) from the erythrocyte by ion exchange chromatography. Guanine—prepared from GTP, isolated after incubation of rabbit erythrocytes with formate C^{14} and 5-amino-4-imidazolecarboxamide riboside—was also found to be extensively labeled. The specific activity was consistently found to be 5 to 10 times greater than the specific activity of the adenine of ATP isolated in the same experiments. Since the GTP concentration in the rabbit erythrocyte was found to be about 10 per cent of that of ATP, the higher guanine specific activity may well be a reflection of this difference in pool size.

It is apparent that the rabbit erythrocyte has the capacity for both adenine and guanine synthesis from formate C^{14} and 5-amino-4-imidazolecarboxamide riboside. The postulated mechanism (fig. 4) involves phosphorylation of 5-amino-4-imidazolecarboxamide riboside by a suitable kinase[6] to form the corresponding ribotide, the compound that has been described as the immediate purine precursor.[5, 8] Formylation and ring closure result in the formation of inosinic acid which may then be aminated via the succinyl derivative by the mechanism described by Lieberman[15] to form adenylic acid. Alternatively, oxidation of inosinic acid to xanthylic acid and amination to guanylic acid may occur by the mechanism described by Abrams and Bentley.[2] The nucleotides formed in this manner may be further phosphorylated to the corresponding triphosphate derivatives.

An alternative mechanism was also considered. If 4-amino-5-imidazolecarboxamide was formed by phosphorolytic cleavage of 5-amino-4-imidazolecarboxamide riboside by a nucleoside phosphorylase similar to the beef liver enzyme described by Korn and Buchanan,[11] a nucleotide pyrophosphorylase could have catalyzed the formation of the corresponding ribotide from 4-amino-5-imidazolecarboxamide and PRPP. Flaks, Erwin, and Buchanan[6] have reported such a nucleotide pyrophosphorylase in beef liver. To test this possibility, the specificity of the rabbit nucleoside phosphorylase with respect to 5-amino-4-imidazolecarboxamide riboside was tested by methods previously described.[16] Evidence which will be reported elsewhere, indicates that 5-amino-4-imidazolecarboxamide riboside will not serve as a substrate for this enzyme.

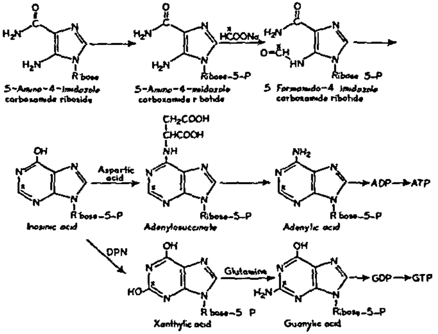
5-Amino-4-imidazole carboxamide riboside
5-Amino-4-imidazole carboxamide ribotide
HCOONa
5 Formamido-4 imidazole carboxamide ribotide
Aspartic acid
Inosinic acid
Adenylosuccinate
Adenylic acid
ADP
ATP
DPN
Xanthylic acid
Glutamine
Guanylic acid
GDP
GTP

FIG. 4.—Probable mechanism for purine nucleotide formation from formate C¹⁴ and 5 amino-4 imidazolecarboxamide riboside.

The capacity of the human erythrocyte for the metabolism of preformed purines via the salvage pathway may be inferred from the work of Preiss and Handler,[20] who showed that an acetone powder extract of the human erythrocyte contained pyrophosphorylases concerned with the reaction of adenine, hypoxanthine and guanine with PRPP to form the corresponding nucleotides.

In the intact rabbit erythrocyte in vitro the utilization of 4 amino-5 imidazole carboxamide in the presence of formate-C^{14} for synthesis of the purine portions of both ATP and GTP, has now been demonstrated although the extent of utilization was not as great as for that of the analogous riboside. The metabolism of 4 amino 5 imidazolecarboxamide to form acid soluble purine nucleotides suggests that this compound may serve as a substrate for a pyrophosphorylase of the rabbit erythrocyte.

The utilization of the purines adenine and hypoxanthine for ATP and GTP formation has also been investigated. The results are summarized in table 2. Although incubation of the rabbit erythrocyte with adenine-8-C^{14} resulted in a considerable labeling of the adenine portion of ATP, the guanine of GTP was

TABLE 2.—*Utilization of Preformed Purines for Purine Nucleotide Synthesis in the Rabbit Erythrocyte*

Incubated*			Isolated	
Compound	Conc.	Activity	Adenine from ATP	Guanine from GTP
	Total µmole	µc	cpm/µmole	cpm/µmole
Adenine-8 C^{14}	15	10	65 000	1 300
Hypoxanthine-8 C^{14}	15	10	8 700	50 000

* Incubated at 37°C for 3 hours in isotonic pH 7.2 phosphate buffer containing glucose (135 µmole).

only labeled to a small extent In view of the relatively small size of the pool of guanine nucleotides it may be concluded that adenine-8 C^{14} is poorly utilized for guanine nucleotide synthesis In marked contrast to the above results when hypoxanthine 8 C^{14} was incubated with an aliquot of the same erythrocyte preparation extensive labeling of the purines of both ATP and GTP was observed Since the specific radioactivities of the adenine and guanine were inversely proportional to the approximate nucleotide pool sizes of these purines it appears that hypoxanthine may be utilized about equally well for these two purines The extensive utilization of hypoxanthine-8 C^{14} further suggests that the poor utilization of adenine-8 C^{14} for GTP synthesis may be due to a limited deamination of adenylic acid to inosinic acid Furthermore the efficient utilization of hypoxanthine for both adenine and guanine nucleotide synthesis suggests that hypoxanthine could be utilized successfully for purine nucleotide formation in the rabbit erythrocyte if it is supplied from another organ such as the liver It should be noted that Abrams and Bentley[2] have indicated that hypoxanthine was insignificantly utilized for the formation of acid soluble purines in extracts of rabbit bone marrow although Abrams and Goldinger[1] had previously reported that hypoxanthine served as a precursor of nucleic acid purines in rabbit bone marrow slices

Several aspects of the biochemical capacities of the rabbit erythrocyte for purine nucleotide biosynthesis have thus been investigated The apparent inability of this cell to carry out the over all synthesis *de novo* of purine nucleotides from the simple precursors in vitro has been demonstrated However the enzymatic reactions constituting the final step of the *de novo* pathway leading to the formation of both ATP and GTP have been shown to occur The ability of the erythrocyte to utilize both adenine and hypoxanthine for purine nucleotide synthesis has also been demonstrated although adenine is a poor precursor of nucleotide guanine

The significance of these studies with respect to the functioning of the erythrocyte and its life span remains uncertain and relates in part to the availability to the red blood cell of compounds such as preformed purines and partially formed purines or their riboside Further extension of this investigation may provide an insight into whether the circulating erythrocyte utilizes these demonstrated metabolic capacities for nucleotide synthesis or whether they represent nonfunctional remnants of the period of immaturity when erythrocyte precursor cell in the bone marrow were engaged in nucleic acid synthesis The demonstration of additional biochemical capacities of the erythrocyte has provided evidence however to contribute further to the demise of the concept of this cell as a biochemically inert bag of hemoglobin

The significance of the results with respect to nucleic acid and nucleotide coenzyme biosynthesis in other types of cells is also obscure It is known however that the erythrocyte is not unique in its limited capacity for *de novo* purine biosynthesis in vitro and in its marked ability to utilize preformed purines extensively for nucleotide synthesis It is possible that one or more centers of *de novo* purine synthesis in an organism serve to satisfy the purine nucleotide requirements of the remainder of the organism by providing preformed or partially formed purines nucleosides or nucleotides which may then be utilized as needed

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Tritium-Labeled Deoxyribonucleic Acid

By ELLEN BORENFREUND HERBERT S ROSENKRANZ
AND AARON BENDICH

THE RECOGNITION that free nucleic acids, when placed in contact with appropriate cells or tissues can cause genetic transformations[5] and induce viral infections[6] or multiple tumors in mice,[7] has greatly stimulated interest in the development of adequate labeling technics to study these phenomena and related metabolic problems Labeled nucleic acids can be prepared biosynthetically, but this method may be quite restrictive For example the preparation via the biosynthetic route of highly radioactive deoxyribonucleic acid (DNA) from human white cells or spermatazoa would require an exorbitant and toxic amount of labeled precursor Since complex molecules such as enzymes and proteins have been rendered highly radioactive by direct exposure to tritium gas (the Wilzbach procedure)[14, 15] it was thought that this method might also be applicable to DNA If this proved feasible it would make it possible to follow the fate in many biologic systems of externally administered DNA

Accordingly, DNA was subjected to the Wilzbach procedure, and a product was obtained in which about one of every thousand hydrogen atoms had been *replaced by tritium It is inconceivable that this level of labeling could have* been achieved by a biosynthetic route

EXPERIMENTAL AND RESULTS

The DNA used was isolated from the white cells of an untreated patient with chronic granulocytic leukemia Many of the properties of the specimen have been described previously ** Eighty five mg of DNA were exposed at 27° C to 2 4 curies of tritium at a pressure of 0 39 atmospheres for 2 weeks † After this treatment, the specific activity was 0 86 mc /mg About 20 per cent of the radioactivity was lost after dialysis for 4 weeks against dilute salt solutions The *dialysis fluid did not show specific absorption in the ultraviolet*

To assess the macromolecular character of dialyzed tritiated DNA (H^3DNA) several types of analyses were carried out

(a) The sedimentation behavior of 0 003 per cent solutions in 0 2 M NaCl was determined[11, 12] in the analytical ultracentrifuge equipped with ultraviolet optics and the integral distribution of sedimentation coefficients were calculated [9] The distributions are shown in figure 1 A decrease in average sedimenta*tion coefficient from 19 3 S for the original DNA to 11 4 S for H^3DNA was ob*

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One of the authors (H S R) is a postdoctoral research fellow of the Alfred P Sloan Foundation

We should like to thank Dr George B Brown for his interest and encouragement

* We are indebted to Dr G di Mayorca for a gift of this specimen

† This procedure was carried out by the New England Nuclear Corp Boston Mass

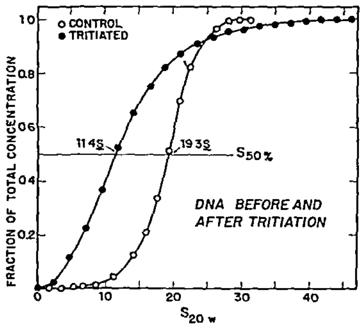

Fig 1—Distribution of sedimentation coefficients of DNA (from human leukocytes) before and after tritiation

served This corresponds to a drop in average molecular weight from 5 2 to 1 29 × 10^6, as calculated from the formula of Doty et al[4] There was also (fig 1) a considerable broadening of the distribution of sedimentation coefficients, from which it can be estimated that there was both aggregation and degradation with the latter predominating This type of change has been observed following the subjection of calf thymus DNA to x rays (10^5 to 10^6 r)[13] However whereas denaturation accompanies the x irradiation of DNA[8] evidence was obtained that no significant denaturation resulted from the tritiation

(b) The evidence that H^3DNA was not any more or less denatured than the original DNA was obtained from measurements of the increases in absorbancy at 260 mμ when solutions were adjusted to pH 3 0 or 13 5 The hyperchromic effects for the original and for H^3DNA were the same within experimental error *These effects have been taken as a measure of the nativeness of DNA (For references and discussion cf refs 8 9)*

(c) Chromatographic analyses were carried out on the cellulose derivative anion exchanger ECTEOLA as previously described[3] The chromatographic profiles of the original and H^3DNA are shown in figure 2 together with the specific radioactivities About 50 per cent of the original DNA was recovered in Region III of the chromatogram i e with eluants of ammonia, which increased from 0 1 to 1 0 M and which contained 2 M NaCl In contrast only about 15 per cent of H^3DNA was recovered in Region III On the other hand, a correspondingly larger percentage of the H^3DNA was recovered in Region I (gradient increase in NaCl concentration from 0 0 to 0 5 M NaCl) and Region II (0 1 M ammonia/2 M NaCl) These differences in the chromatographic profiles (fig 2) can be correlated with the differences in the distribution of sedimentation coefficients (fig 1) A positive correlation between sedimentation coeffi

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Discussion of DNA Transformation

F Chargaff Dr Friedkin how would you explain the situation in which a thy*mine-deficient F coli becomes capable of* making thymine once it is infected by a phage particle? Would you assume that this is an induced enzyme formation?

M Friedkin I think Seymour Cohen *should be here to answer that question* There exists an *E coli* mutant which is incapable of making thymine If this *E coli* mutant is infected by a particular phage this phage apparently transfers information *to the E coli, which now allows it to make* thymine for new phage DNA The point is that an enzyme emerges which catalyzes the methylation of deoxyuridylic acid to thymidylic acid This enzyme was previ*ously absent in the E coli mutant*

F Chargaff Dr Bendich in connection with these experiments on labeled thymine which show this unusually high ratio (compared to the others is that result *repeatable for a given species of DNA* or is that more or less a random finding?

A Bendich We have studied tritium labeling only with a particular DNA which had been isolated from the leukocytes of a patient with *chronic granulocytic leukemia* We haven t learned yet whether or not the different fractions of the DNA all show the same phenomenon What we have done to date is to examine the distribution of tritium in the bases of all DNA

W Damfshek Dr Bendich I found your talk very stimulating and provocative Would you expand a bit on the tumor stimulatory effects of certain nucleic acid moieties?

A Bendich There are probably a dozen examples of infective nucleic acid For example one can prepare from a partially purified polio virus an RNA devoid of virus and this RNA will infect HeLa cells in *tissue culture from which one can then re*cover virus[1] The most recent example is one by Cheng who isolated an infective RNA from the brains of mice infected with Semliki Forest virus[2] I think the first in *the mammalian series was by Wecker and* Schaefer[3] and in the plant series by Gierer and Schramm[4] There are some other experiments dealing with (DNA) preparations which appear to be carcinogenic[5,6]

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Discussion of Radiation Effects of Cellular Labels*

W L HUGHES It might be expected that incorporation of a β emitting isotope within the radiosensitive volume of the nucleus would produce radiobiologic effects Evidence for the lack of radiation effects thus appears important in any interpretation of labeling data

Radiation effects in the form of chromosomal aberration were first observed by McQuade and Friedkin using C^{14} labeled thymidine with a specific activity of 3 curies per mole[1] With the availability of H^3 thymidine of specific activities up to 3000 curies per mole, more dramatic effects should be observed and using such material we have been able to kill cells in tissue culture[2] Inhibition of cell proliferation occurred when the intranuclear concentration of tritium approached that concentration of tritium oxide necessary to produce similar effects While there is no evidence here of any unpredicted toxic effects the margin of error, which includes both the measurement of nuclear volume and the precise amount of intranuclear tritium is large Consequently agreement much closer than an order of magnitude is probably fortuitous

Taylor has found an 8 per cent excess of chromosome breaks in bellavalia root tips when the nuclear content of tritium was sufficient to produce 60 disintegrations per nucleus during the interval investigated (36 hours)[3]

The β particle produced by a disintegration of H^3 has an average energy of 5 700 electron volts Since this has an average range of only 1 μ in tissue most of the energy will be absorbed within a very small volume including its source If this energy is all absorbed in 100 μ^3 of tissue it will produce an average dose of 1 r to this volume Estimating the volume of the bellavalia nucleus to be 200 μ^3 we see that 60 disintegrations would provide about 30 r to the nucleus assuming complete absorption in the nuclear volume Taylor says that the frequency of chromosomal aberrations observed is that to be expected from a single exposure to 50-100 r of x rays[3]

Again the excellent agreement is possibly fortuitous in view of the large uncertainties attendant upon the estimates of autoradiographic efficiency and of nuclear volume Nevertheless both experiments serve to suggest that rough estimates of radiation dose may be made from grain counts and measured nuclear volumes so that if information on the radiosensitivity of the cell is available labeling levels may be so chosen as to avoid this complication Alternatively, it would also seem usually possible to ' spot check ' occasional points in any analysis using lower levels of radioactivity and longer autoradiographic exposure times to prove that the results are not radiation-dependent

M FRIEDKIN Dr McQuade and I have continued our studies on chromosome aber

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* The problem of possible radiation effect on labeled or adjacent cells is an important consideration in the study of cell proliferation Time did not permit formal discussion of this aspect at the conference However the participants have been invited to comment on the general statement about radiation submitted by Dr Hughes Most of the comments were on tritiated thymidine which presents a special type of problem in that the energy delivered with each disintegration is confined to a small area of the nucleus Isotopes other than tritium present problems more nearly approximating those encountered with external radiation This is particularly true for Fe^{59}, on which Drs Robertson and Bond have commented —*Ed*

DISCUSSION TABLE 1.—*Radiation Effects of Thymidine Labeled with Carbon 14 and with Tritium*

(See Exper. Cell Res. *11*, 249, 1956, for details of scoring)

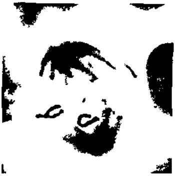
Treatment of onion roots for 19 hr	Micromoles per ml	Microcuries per ml.	Number of meristems	Cells scored	Aberrant cells (%)	Micronuclei per meristem
Water	—	—	11	558	0.35	5.0
Nonradioactive thymidine	0.42		12	693	1.58	7.8
2 C^{14} thymidine	0.42	1.26	11	475	4.20	24.0
Methyl C^{14} thymidine	0.42	1.26	10	452	10.2	29.6
Tritiated thymidine	0.42	1.26	12	667	4.94	18.4
Tritiated thymidine	*0.025*	22.0	*12*	*269*	*99.3*	*too numerous to count*

rations in the onion root tip caused by radioactive thymidine.[1] Our initial investigations were with thymidine labeled with carbon 14 in the 2-position. In our present work we have compared the effects of isotopes located in different parts of the thymidine structure: carbon 14 in the 2 position, carbon 14 in the methyl group, and tritium presumably in the 6 position. Our preliminary data show an astonishing radiation effect produced by H^3 thymidine of high specific activity (890 curies per mole). Almost every cell showed signs of radiation damage (Discussion table 1 and fig. 1). We are very much interested in the genetic implications of these chromosomal changes.

J. S. ROBERTSON: In considering the radiation dose from individual disintegrations, it must be kept in mind that the units of radiation dose are intensive, like temperature, rather than extensive, like heat. The meaning of average doses taken over a volume which is not uniformly irradiated may therefore be questioned. In cell nuclei irradiated with tritiated thymidine there will be some cubic micra getting over 100 rad and others getting close to zero rad. Until more is known about the fine structure of the distribution, however, it is necessary to make comparisons on the basis of average dose, as Dr. Hughes has done.

L. D. HAMILTON: Dr. M. L. Fidinoff and I have discussed the question of possible radiation damage to cells from tritiated thymidine and it seems highly likely to us that this effect would not be significant in short term (several hours) tracer studies with mammalian cells. In the first experiment which M. Errera and I did with teased lymph node preparations we were anxious to get radioautographs quickly and incubated cells for four hours in 1 ml. volume with 100 microcuries of tritiated thymidine. At the end of this time the few cells that had taken up thymidine were ghosts, only the compact grains corresponding to their nuclei with a faint trace of cell remnants remained. We calculated that these nuclei received 30–40 rad and that this dose of radiation under these circumstances produced acute cytolysis.

However, in experiments of longer duration after several generations of mammalian cell growth and proliferation, cells may show delayed radiation effects even at relatively low specific activities. Therefore we agree emphatically with Dr. Hughes that in long term studies it becomes impor



DISCUSSION FIG. 1.—Chromosome aberrations in a Feulgen stained cell of the meristem of an onion root treated with *tritiated thymidine of high specific activity* (890 curies per mole).

tant to consider check experiments at, say, one-tenth the specific activity, to see if the biologic conclusions are in line with those from the higher activity tracer. This would be a useful test for radiation damage.

W. PLAUT[*]. It is possible to approach the question of potential radiation damage in tritium labeled chromosomes from grain counts in autoradiographic preparations by way of total ionizations per chromosome and Lea's estimates[4] of the probability of chromatid breaks in terms of ionizations. When this is done (see reference 5 for details) one obtains estimates of from 50 to several hundred breaks per chromosome in a 12 hour incubation period for *Vicia* chromosomes in a typical tritiated thymidine experiment. This estimate is in very poor agreement with the frequency of cytologically detectable aberrations in anaphase and telophase of tritium labeled cells. However, the comparison of expected breakage with cytologically detectable breakage suffers from a serious difficulty. Classic methods of breakage detection are sensitive only to breaks and recombinations involving chromatids or chromosomes. These methods are not adequate for the investigation of disturbances below the structural level of the chromatid. The short range and high ionization density of the tritium β particle suggest that the latter type of disturbance is the most likely and that chromatid or chromosome interchanges are relatively rare. While exchanges at the subchromatid level would not necessarily constitute biologic damage (the cell may be as insensitive to these as are our detection methods) they would interfere seriously with autoradiographic studies on chromosome duplication and segregation.

E. P. CRONKITE. The general problems concerned with analysis of radiation effects by the intranuclear label tritiated thymidine have been discussed previously.[6] By using the customary criteria of radiation injury in radiosensitive tissues (pyknosis, atrophy, obvious mitotic arrest and aberrations, etc.) we have not seen any clear-cut evidence of radiation injury in radiosensitive tissues of the mouse, guinea pig, dog or rat with doses up to 1 μc per gram (specific activity 1.9 curies per millimol). Up to 0.4 μc per gram has been given to terminal patients without obvious cytologic effects. However, very adequate autoradiographs can be obtained with 0.1–0.2 μc/gram in human beings with exposure periods of 30 to 60 days.

Johnson et al. are studying the radiation effects of H^3 thymidine on spermatogenesis of the mouse. Depletion of the spermatocytes is barely detectible at a level of 0.5 μc/gram. After higher doses the effect increases and at a level of 20 μc/gram the effect approximates that of about 40 rads of external x-ray.

It is quite clear that the dangers of H^3 thymidine in terms of potential carcinogenic effect and genetic hazards have not been evaluated adequately as yet. The acute effects after large doses are minimal. The volume of bone marrow nuclei assuming spheres will vary from 100 μ^3 (radius about 3 μ) to 1300 μ^3 (radius about 7 μ). The volume of the cells that perpetuate hemopoiesis is not certain but probably varies between 200–500 μ^3. Using Hughes' value of 30 r to a 200 μ^3 nucleus from 60 disintegrations, one can estimate doses to various primitive hemopoietic cells. Grain counts over the larger nuclei in a 30 day exposure are as high as 90 after a dose of 0.1 μc/gram to patients. If one assumes an autoradiographic efficiency of 1.0 per cent, there are about 300 disintegrations per day. If the volume is the same, the average maximum dose to the *nucleus* will be of the order of 150 r the *first day*. Since cell division in these cells occurs every 24 hours or even more rapidly in some cases, the dose is rapidly cut in half for subsequent time periods. In the above computation, dose was estimated on *maximum* grain count and a low autoradiographic efficiency factor which overestimates the dose. For the first seven days (assuming 7 mitoses) the maximum nuclear dose would be of the order of 300 r. On the basis of mean grain counts it would be of the order of 70 to 100 r during the first week for the primitive progenitor cells as defined in my opening paper of this conference. It is quite evident that the *average dose* varies with autoradiographic efficiency and nuclear volume of the cell which perpetuates hemopoiesis. Since neither is known, these numbers have little real meaning. Lastly, the *average dose* has no real meaning since only the dose to vulnerable structures is of significance in deter mining the effect There is no doubt that tritiated thymidine can produce effects but these effects must be determined by empiric studies not computation Further studies are needed on the potential carcinogenic and genetic effects of H^3 thymidine

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[*] Dr. Plaut's comments were submitted by Dr. V. R. Potter.—*Ed.*

L G Lajtha As mentioned by Dr Hughes the average range of H^3 β particles is about 1 μ in unit density material If one calculates the dose of radiation delivered to a sphere of 1 μ diameter (in which most of the energy of a single emission will be dissipated) one gets the figure of 168 rads The volume of an average bone marrow cell nucleus is about 500 μ^3 any H^3 emission may hit any one μ^3 of this volume It is obvious that it will be meaningless to speak of radiation dose per nucleus as the dose will be singularly uneven within the nucleus and subsequent disintegrations may even affect the same $1\mu^3$ volume However any $1\mu^3$ volume which received 168 rads has received a significant hit The biologic effects will depend on whether this volume contained a vulnerable structure and whether a damage to this structure is quickly reparable or not

It is feasible—in fact to be expected—that if calculated on the dose per nucleus the H^3 rads/nucleus produce less visible chromosome damage than equivalent rads/nucleus of x rays—but this is simply due to the fact that one cannot speak of rads/nucleus when dealing with H^3

Nevertheless certain vulnerable volumes in the nucleus may receive 168 rads and it will be left to chance *which* $1\mu^3$ of the nucleus will contain *what* vulnerable structure

The two tests which may give perhaps the best measure of damage due to intracellular H^3 would be (a) number and size of colonies growing out of a population of of H^3 thymidine containing cells (Puck method) and (b) study of mutations through several generations

C G Craddock It would seem that an opinion as to the toxicity of tritium labeled thymidine will have to await long term studies of induced genetic abnormalities Toxicity as measured by actual cell death impaired DNA synthesis chromosomal breaks etc must reflect severe damage and the absence of such changes would not absolve such a strategically localized isotope of serious long term effects in my opinion Dr Hughes makes a key statement when he says that rough estimates of radiation dose may be made from grain counts and measured nuclear volumes so that if information on the radiosensitivity of the cell is available labeling levels may be so chosen as to avoid this complication The information to which he refers is not available for most normal mammalian tissues

L F Lamerton Dr Hughes remarks are very encouraging to those of us who are using tritiated thymidine as a tracer His observations suggest that some of the estimates that have been made of the extremely damaging effect of tritiated thymidine by virtue of its intimate relationship with the DNA molecule are unjustified It would be very interesting to know whether the distribution of types of chromosome abnormalities in a given material are the same whether produced by incorporating tritiated thymidine or by external radiation

H M Patt I should like to suggest that cognizance be taken of the recent report by W R Guild[8] Guild indicates that under certain conditions thymidine could deliver a dose to chromosomes some 50 to 5000 times greater than might be obtained from an equal amount of tritiated water activity

V P Bond As Dr Hughes has indicated the problem of calculating the dose from tritiated thymidine incorporated into the nucleus is an extremely difficult one Because of the highly selective localization of the radiation the considerations that ordinarily apply to the calculation of radiation dose from isotopes cannot be used directly Depending upon the assumptions made an order of magnitude or greater difference in the calculated value of rad dose delivered on a microscopic basis can be derived Thus little basis can be found for comparing doses so calculated with the rad doses calculated or measured for x radiation or for isotopes such as P^{32} and Fe^{59} that are used extensively in research and clinical work and which localize in highly sensitive systems It appears likely that a comparison with different radiation of total *events* within the cell nucleus comparable to that produced by the disintegrating tritium atom might be another reasonable approach to seeing if tritium fixed within a cell nucleus is indeed as hazardous compared to other commonly used isotopes as the calculated rad doses might indicate

It thus seems clear that at present we must rely on isotope dose levels delivered to biologic systems, and the resultant effects obtained. Dr. Hughes has indicated that Dr. Drew, Dr. Painter, and he have observed effects from tritium labeled thymidine added to tissue culture and delayed or inhibited division has been observed at doses as low as .02 microcuries per milliliter of culture medium. However, these results cannot be carried directly to the mammal in which the material is given intravenously, because the availability times are widely different *in the two situations. In addition the specific activity of the material used will make a considerable difference in the amount incorporated.* It thus seems necessary to go directly to the mammal if one wishes to obtain an isotope dose–effect relationship. Dr. Johnson in our laboratory is studying the effects of such incorporated tritium labeled thymidine on the sensitive spermatogonia in the mouse.[7] He obtains definite effects at 1 microcurie per gram injected intravenously and equivocal effects at 0.5 microcuries per gram. In our studies of human beings we have used less than 0.5 microcuries per gram and it is unlikely that the tritium has perturbed the systems studied to any appreciable degree. *In my paper I indicated that one possible explanation for the rapid fall in* the erythroid precursor series was a radiation effect by the tritiated thymidine. I wish to point out again however that this is only one possible explanation and that it by no means proves that the tritiated thymidine had perturbed the system.

J. S. ROBERTSON Iron 59 decays with a 46.3-day half time by β γ emission except that in 0.3 per cent of the disintegrations a 1.560 MeV β only is emitted.[9] The β s have ranges in tissue of many cell diameters, *so although it is true that in the bone marrow the iron localizes predominantly in* the erythroid series in calculations of the radiation dose a uniform distribution of absorbed energy in the bone marrow can be assumed. A correction for energy absorbed in bony trabeculae is applicable,[10] and in the mouse where the β particle range exceeds the marrow cavity radius a small correction for β particle escape applies.

Taking 0.120 MeV as the average β energy (reference 11 page 899) *for 1 μc/*gram, the usual calculation, using $R_\beta = 51.2\bar{E}_\beta C$, gives a figure of 6.13 rad/day, or 4.91 rad/day with the 0.8 correction for energy absorbed in trabeculae. Using as the bone marrow weight 2.1 per cent of the body weight, a dose of 100 μc/Kg of body weight would become 4.75 μc/Gm of bone marrow if 100 per cent of the iron were to localize in the bone marrow. Such a high degree of localization may be an unrealistic assumption but a concentration of only 50 per cent in the marrow or 2.8 μc/Gm, would give an initial dose rate of 13.7 rad/day. *Interpreting Hennessy and Huff s data[12] on iron uptake in red cells of rats as meaning that iron has a half time in* the marrow of only one day the β dose to the marrow becomes 19.8 rad. If the half time in the marrow of larger animals is two days the β dose becomes 39.6 rad.

The gamma ray dose is more difficult to calculate precisely for the peculiar geometry of the bone marrow. But with 43 per cent of the gamma rays having 1.289 MeV and 57 per cent having 1.098 MeV[9] the gamma contribution can be expected to be substantial in large animals. The Fe^{59} γ ray dose constant, Γ, is 6.13 cm^2 r/mc hr (reference 11 page 758). *Assuming uniform distribution in the whole body the usual formula $R_\gamma = 10^{-3}\ C\ \Gamma\ G$ yields using $G = 150$ for the geometry factor a dose* rate of 2.21 r/day at the midline for a whole body concentration of 100 μc/Kg. The dose to the marrow is at least half of this or 1.1 r/day for uniform distribution and may be somewhat higher when a large fraction of the iron is concentrated in the marrow although in the latter case a smaller geometry factor offsets the higher concentration value. For small animals the geometry factor diminishes to 50 or less and the dose rate is proportionately smaller.

Using the physical half time of 45 days as the effective half time for Fe^{59} in the body the integrated γ dose to the marrow comes to about 65 rad for large animals and 15 to 25 rad for small animals. (NBS Handbook 52[3] gives 27 days as the effective half time but more recent clinical data[14] indicates that a longer biologic half life obtains.)

According to these calculations the combined β and γ radiation dose to the bone marrow therefore ranges between 40 rad and 100 rad for an initial whole body con centration of Fe^{59} of 100 μc/kg depending on the size of the animal

F Stohlman Jr In considering Fe^{59} dose to the marrow there are several points worthy of mention The β dose delivered to the marrow will vary with the emergence time of the red cell the developmental stage at which Fe enters the red cell and the percentage of injected iron incorporated by the red cell precursors Thus in the rat where pronormoblasts take up little iron the Fe^{59} marrow time is substantially less than in dog or man where earlier precursors are heavily labeled This is reflected in a peaking time of 48–60 hours in the rat in contrast to 96 hours in dog and man Moreover in the rat only about 50 per cent of the injected dose of radioiron eventually emerges in the mature red cell whereas in dog and man the figure is 70 to 90 per cent These considerations may lead to a two- to fourfold difference in the marrow dose of different species

The dose arising from gamma emissions continues to be delivered to the marrow after emergence of Fe^{59} from the marrow in mature red cells Nevertheless due to dilution of label one can follow no more than about three generations of the erythroblastic progeny Thus the life of the autoradiographic study is of the order of seven days or less Accordingly for calculation of dose to the population under study a value of 0 098 (the fraction delivered per week[15]) × total delivered dose would appear appropriate

W L Hughes Generally there appears to be concurrence with my remarks on radiation effects from tritium Friedkin s data suggest that the radiotoxicity in onion roots also may be interpretable in terms of nuclear concentration The tremendous effects observed with H^3 thymidine of high specific activity are in line with our own observations in tissue culture We find significant killing at the much smaller concentrations of 0 02 microcuries/ml of culture medium Whether this is a result of greater radiosensitivity of the HeLa cell or only of greater permeability cannot be discerned from the existing data Of course these results cannot be extended to whole animal studies since the relevant data would be the intranuclear concentration While this has not been quantitated in the HeLa cell experiments autoradiographs show that it is obviously many times that attained in in vivo labeling studies

The problem of nonuniform distribution of radiation from tritium has to be considered from several points of view First within the dose range we are considering the amount of tritium is great enough so that statistical fluctuations in the decay process will not appreciably alter the dose per cell Therefore radiation effects resulting from single hits such as mutations and perhaps carcinogenesis which depend on the total number of ionizations per cell should be equivalent for the same accumulated dose whether from x rays or β particles However effects dependent upon multiple hits might reasonably be expected to depend on the distribution of these both in time and space For such effects the clustering of ionizations produced by tritium might markedly alter radiosensitivity That tritium has shown no large differences in terms of relative biologic effectiveness suggests that the damage resulting from hits is additive and repair processes are not important

While the nature of the radiation does not seem too important its concentration within the radiosensitive volume certainly does One might expect that the effects we have noted for intranuclear concentration might be further extended to intrachromosomal concentration[6] The fact that such effects have not been observed may merely mean that during most of the cell cycle the chromosomes are so diffuse and intermeshed that any β particle passes thru several of them The hypothetic effects postulated by Plaut to justify his mechanism of chromosomal duplication seem highly improbable to me

In conclusion it must be said that the whole radiation story is quite fragmentary and much more data is needed Possible complications in the interpretation of tracer experiments due to the radiation used seem generally susceptible to direct estimation The possible health hazards from ingestion of tritiated compounds depend largely upon retention and reutilization of the label While Guild s estimates[9] of this hazard are probably extreme they nevertheless serve as a timely reminder centration of Fe^{59} of 100 μc/kg depending on the size of the animal

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Section III. Studies with DNA Labels

E. P. Cronkite, *Chairman*

DNA P^{32} Studies of White Blood Cell Formation, Distribution and Life Span

By DANIEL L KLINE

SEVERAL YEARS AGO when it was established that radiophosphorus is incorporated into the deoxyribo e nucleic acid (DNA) of cells at the time of their formation, and remains stably bound throughout the lifetime of the cell we began our investigation of the life span of the white blood cells In general the work progressed as anticipated and yet today many experiments later we are less sure than ever of how long the white cells live

Our first experiments consisted in the oral administration of P^{32} as inorganic phosphate to a group of seven patients who did not exhibit any signs of hematologic disorder[1,2] Samples of blood were obtained periodically for three weeks The white cells were collected and the specific activity of the DNA phosphorus (DNA P) was determined The Schmidt Thannhauser procedure the basis of the method we used for DNA isolation has been criticized[3] but we have never been able to detect activity in unlabeled DNA which was isolated from a variety of tissues in which the other phosphorus fractions were heavily labeled The newer method is undoubtedly superior as judged by sensitive chemical tests but under the conditions of our experiments our analyses of DNA appear to be satisfactory

In figure 1 each point expressed as counts per minute per mg DNA P represents the average obtained from five to seven patients The total and differential counts of these patients remained within normal limits throughout the experimental period Little activity was found before the fourth day at which time there was a sharp increase in circulating DNA labeled leukocytes This high level was maintained until about the tenth day when activity decreased abruptly but did not disappear entirely In these experiments only two or three points were obtained for each subject between the fifth and tenth days Although the average values resulted in an apparently smooth plateau subsequent analysis has shown that this is rarely found in an individual After the abrupt drop in activity the lower level which was reached persisted for a long time at least three weeks

The equations used by Shemin and Rittenberg in their classical study on the

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life span of the red blood cells[21] were employed by us to calculate the point of the maximal rate of disappearance of labeled cells (fig 2) This was found to occur 13 days after isotope administration and about 9 days after the sudden influx of labeled cells into the circulation Although we recognized the limited interpretation which could be based on these data, it seemed that the interval between the points of sudden increase and rapid fall delineated a minimum average life span for leukocytes which was very much longer than the generally accepted value of a few hours that had been inferred from nonisotopic experiments

These measurements had been made of the leukocyte population as a whole Dr Ottesen, in Denmark, kindly provided us with the details of his method for separation of lymphocytes from granulocytes and we also learned that he had carried out experiments similar to ours several years earlier, although they were not published until later[19] Using a modification of this technic[11] we studied the appearance and disappearance of labeled lymphocytes and granulocytes in a series of polycythemic patients who received P^{32} [7] In figures 3 and 4 the close resemblance of these curves to those that had been obtained earlier is apparent Note that even the averaged curve of the lymphocytes shows distinct peaks at about the fifth and ninth days

In considering the possible reasons why nonisotopic measurements give short life spans (hours) as compared to the P^{32} experiments (days), a review of the literature reveals that the life span values fall into three groups depending on the methods used

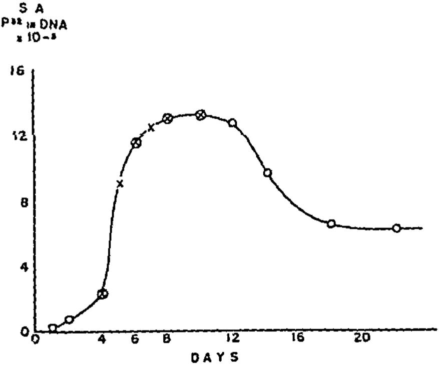

FIG 1 —Specific activity of DNA phosphorus of circulating leukocytes in man

1 Very Short Life Span

(a) These methods are based on the persistence of cells introduced from one animal to another The cells may be labeled or unlabeled This category includes cross-circulation[13] and transfusion[25] experiments The cells introduced disappear within an hour or two, a finding which is most easily explained by the segregation of cells in certain capillary beds, particularly in the lung The astounding capacity of this segregation mechanism has been effectively demonstrated in the heart lung preparation [1] By measurement of arteriovenous differences [2] and by the use of in vivo tagging with fluorescent dyes [15] this mechanism has been shown to occur even when the cells are not introduced from without The possibility must also be considered that the release of new cells may be inhibited by the introduction of white cells into the circulation

(b) Replacement of a depleted cell population These experiments include parabiosis in which one partner is leukopenic [23] cross-circulation under similar conditions [9] and leukophoresis [5] The extensive leukophoresis studies from Craddock's laboratory, which were performed with and without isotopes and Hollingsworth and Finch s cross circulation experiments have provided solid experimental confirmation of the existence of truly enormous white cell reserves in the body It is obvious as these authors recognized that the time required to replace a white cell deficiency is not a life span measurement

(c) Cannulation of the thoracic duct[26] yields a life span of 12 hours

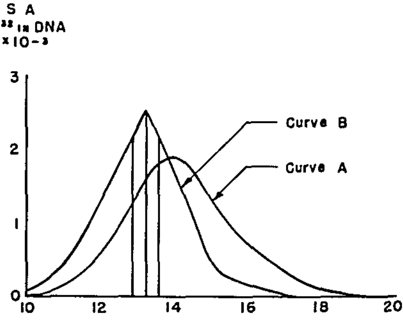

Fig 2 —Curve A Change in specific activity of DNA phosphorus of circulating leukocytes in man from tenth to twentieth days Curve B Curve A corrected for inflow of labeled cells

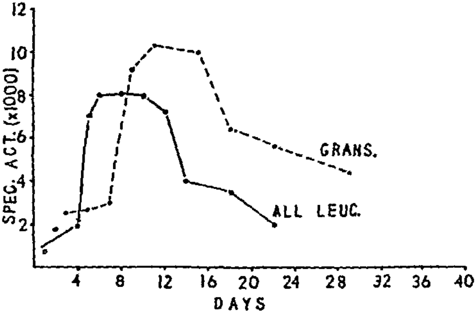

Fig 3—Specific activity of DNA phosphorus of circulating granulocytes in polycythemia vera and normal leukocyte curve

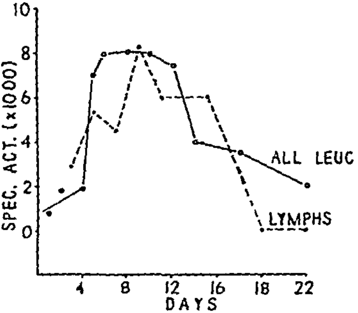

Fig 4—Specific activity of DNA phosphorus of circulating lymphocytes in polycythemia vera and normal leukocyte curve

2 Intermediate Life Span

In these experiments, the cells were tagged in vivo with fluorescent dyes[14] or diisopropyl fluorophosphate (DFP).[3] All but a few of such labeled granulocytes disappeared from the circulation within 48 hours. Events within the leukopoietic tissues may explain the longer blood survival time found in these studies than is seen when labeled cells are injected into the circulation

3 LONGER LIFE SPAN

When cells are labeled in vivo with radioisotopes that become firmly bound in stable cellular constituents labeled granulocytes are detectible in the circulation for 5 to 13 days after P^{32} [1] [18] [19] 13 days after Fe^{59} [20] Cr^{51} [16] or S^{35} and 9 days after C^{14} guanine or adenine administration [8] The picture in regard to lymphocytes varies considerably for reasons which will be discussed Two types of lymphocytes with life spans of 3 days and 2 or more months respectively have been postulated[5] [19] [20] in studies in which P^{32} Fe^{59} and C^{14} were used *Intermediate values have also been obtained* [4]

It is not difficult for one who has used isotopes to point to overwhelming evidence that most of the nonisotopic procedures might more properly be termed deficiency replacement time or blood clearance measurements However this dismissal of other technics by no means constitutes proof that the isotopic methods give a more exact measure of the actual time of survival of the leukocytes in the body In recognition of this fact the remainder of this presentation is devoted to a consideration of the difficulties in the interpretation of P^{32} measurements of the life span of leukocytes

In an attempt to reconcile the longer life span measurements with the shorter values we decided to concentrate on the lymphocytes since the duct collection method of Yoffey [6] had yielded a life span value of 12 hours in his and other laboratories and it is not a deficiency replacement or blood clearance measurement Although the number of lymphocytes carried to the blood by way of the thoracic and right lymphatic ducts in approximately 12 hours is equal to the total number of lymphocytes found in the blood stream at any one time we found after P^{32} administration and isolation of DNA from the blood lymphocytes in the rabbit[4] that a large number of circulating labeled cells were detectible for at least 12 days (fig 5) In addition we analyzed mesenteric lymph nodes and to our surprise found that labeled cells were present in these nodes for an equal length of time (fig 5)

The explanation of the isotope curve of these labeled lymphocytes in lymph nodes presents a challenging problem since the previous interpretation of isotopically determined life span measurements is thrown into jeopardy by this finding It is possible that the cells obtained from the blood had been newly added to the circulation perhaps even twice a day in agreement with the duct collection data As yet we have no evidence that positively rules out this interpretation

How can this finding be explained? The inorganic P^{32} of the blood declines logarithmically Since this fraction appears to be a direct precursor of DNA the residual P^{32} of the blood or of lymph nodes (unpublished observations) cannot account for this phenomenon A recirculation of lymphocytes has been proposed [22] but the experimental evidence is largely negative The recent work of Yoffey Everett and Reinhardt with labeled thymidine[27] is particularly discouraging to this hypothesis

On reflection, it occurred to us that the finding that DNA labeled cells persist in the nodes should not have come as a surprise Approximately 3 Gm of lymphocytes (the amount calculated to be in the circulation) are transported via the main lymphatic ducts every 12 hours. This involves only about 7.5 per cent of the mature lymphocytic cells of the lymph nodes.[27] If these cells were released according to their age, we should not expect to find labeled lymphocytes in the circulation in appreciable numbers until about seven days after isotope administration. Instead, in the rabbit, labeled blood cells are detectible within a few hours and in man within two days, with the peaks and declines that have been shown (figs. 4 and 5). With random mixing in, and release from the nodes, labeled cells should have appeared and maintained a steady plateau in the blood. Consideration of these facts leads to the conclusion that a part, perhaps the major part, of the newly formed lymphocyte population is discharged from the nodes soon after their formation and that the remainder is retained for varying lengths of time, depending on the needs of the organism. While this would place our findings in harmony with the thymidine data of Yoffey et al.[27] we are at a loss at present to explain how the nodes acquire and maintain a high level of activity if the lymphocytes do not recirculate.

The presence of a partly labeled pool in the nodes could explain several puzzling phenomena: the biphasic blood lymphocyte curve observed by Fichtelius,[4] which was abolished by bleeding; the persistence of labeled lymphocytes which can be detected at low levels for very long times after the administration of radioisotopes;[8, 19, 20] and the failure of isotope curves to return to a zero baseline.

It cannot be concluded with certainty that the lymphocytes have a long life span within the lymph nodes. Several laboratories have described a three day blood peak for lymphocytes. Our data show that this phenomenon occurs within the nodes, and suggest the possibility of turnover within the lymph node itself.

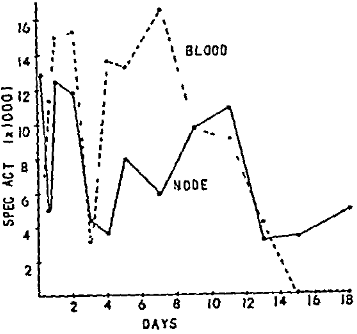

FIG 5.—Specific activity of DNA of lymphocytes in lymph nodes and blood in the rabbit

Both the human and rabbit blood lymphocyte curves show several peaks The cells that comprise the reserve pool, therefore may be in a dynamic state that could involve the reutilization of nuclear and perhaps other-[9] material as has been suggested most recently by Hamilton[8] The side by side occurrence of lymphocyte formation and disintegration within nodes has been described but not explained[9] On the basis of these uncertainties one must be wary of the conclusion that isotope studies have proved that any lymphocyte has a long life span

From histologic tissue culture and isotope data Osgood has calculated that there is a ratio of approximately 400 lymphocytes outside the blood stream exclusive of lymphopoietic organs to each circulating lymphocyte[17] If the mature lymphocyte remains longer than three days in lymph nodes and if the life span after release has been overestimated in isotope studies a drastic decrease in the extra to intracirculatory ratio would result Life span estimations and calculations of the size and state of the extravascular pool are intimately related proponents of a long life span point to a large pool in support of this idea and vice versa *While there are undoubtedly suggestions that white cells leave the blood* stream there has been no direct experimental demonstration of what size such a pool might be or if these cells are in equilibrium with the circulating cells After leukophoresis replacement granulocytes came from the leukopoietic organs[5] When granulocytes were collected after the intraperitoneal injection of Locke's solution the cells obtained were heavily labeled[4] indicating that they had not been diluted in a large extravascular pool In our laboratory Mr Hinckley investigated the replacement of lymphocytes after hydrocortisone injection in the dog and rabbit (table 1) In both species shortly after P^{32} administration or seven days later the cells found after the animals had recovered from the induced lymphopenia came from the nodes or other sites of formation and not from an extravascular pool While these experiments do not rule out a large extravascular pool if it exists it has been very difficult to demonstrate that *extravascular cells can return to the blood stream in either acute or chronic* states of leukopenia The existence of a large number of leukocytes that were not in equilibrium with the blood cells would of course further undermine the

TABLE 1 —*The Appearance of Labeled Lymphocytes in the Blood Following Hydrocortisone Induced Lymphopenia*

Species	Time after P^{32}*	Time after hydrocort.†	Lymphocytes	
			Blood	Node
			c p.m. mg DNA P	
Rabbit	8 hrs	6 hrs	5 100(4)	1 40(5)
	8 hrs	—	00(4)	5 090(5)
Rabbit	7 days	8 hrs	4 750(4)	4 380(5)
	days	—	350(4)	3 050(5)
Dog	2 days	10 hrs	6 440(1)	12 000(1)
	2 days	—	4 1 0(1)	4 000(1)

* 100 μc /kg I P

† 0.3 5 mg hydrocortisone/kg I V

validity of life span measurements based on blood sampling. It may be attractive and even temporarily useful to adopt a theory when it accords well with the known data, but it can also be dangerous when, by circular reasoning, a long life span is hypothecated because of a large body pool, and the presence of the large pool is inferred from a supposedly long life span.

It appears to us that the interpretation of lymphocyte life span and cell distribution data from isotopic studies will be placed on firm ground only when the events occurring in lymph nodes are worked out experimentally, and when the size and dynamic state of the extravascular pool is ascertained by actual measurement. We believe that with tritiated thymidine through direct and radioautographic studies, in combination with P^{32} measurements, it will be possible to clarify the lymph node picture and possible to develop a method for the sampling of extravascular cells in order to obtain a measurement of the state of equilibrium of these cells with those of the blood stream. Thus the true life span of the leukocytes may be estimated.

SUMMARY

Most nonisotopic estimations of the white cell life span, which give values of a few hours, are actually measurements of cell replacement or blood clearance times. In vivo labeling with dyes or DFP gives a granulocyte blood survival time of about 1 day, but more information is needed before firm conclusions can be reached. The persistence of circulating lymphocytes in which DNA has been labeled with P^{32} or other radioisotopes that are incorporated into stable cell constituents at the time of cell formation is not a measure of life span or blood survival time, but appears to be a reflection of events going on in the nodes. Thoracic duct collection data and DNA-P^{32} analyses of lymph nodes, together with the known size of the pool of mature lymphocytes in lymph nodes, show that mature cells may remain in the nodes for long periods of time, either in a stagnant or a constantly renewing state. As a corollary to this view, calculations based on a 3-day retention of mature lymphocytes in lymph nodes, followed by 27 days in the circulation, may grossly overestimate the size of the extravascular and extralymphopoietic lymphocyte pool.

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Carbon^{14}-Labeling of DNA in Studying Hematopoietic Cells

By L. D. HAMILTON

THE MECHANICS of the presentation of data can unconsciously distort interpretation. A study of the kinetics of cellular proliferation must skirt the pitfall illustrated in figures 1 and 2.

We have studied the incorporation of C^{14} labeled precursors into the nucleic acids of human leukocytes in order to discern biosynthetic pathways of nucleic acid synthesis and function.[3,5] These pathways have assumed prominence as especially good targets for the chemotherapy of leukemia. The more that can be learned about these pathways, the more rational the chemotherapeutic procedures.[2] Incorporation of purines and pyrimidines by man is accessible to experimentation, because man can incorporate these into his nucleic acids, thus bypassing synthesis from smaller precursors.[3,5] (It is to be noted that unlike in insects and many microorganisms this bypassing has not reached the stage of absolute dependence on exogeneous purines and pyrimidines.) Studying the kinetics of DNA and RNA in human leukocytes is thus a by-product of our studies on nucleic acid synthesis. Data obtained from this technic contribute nicely to analysis of RNA and DNA formation and turnover.

The diversity in patterns of purine utilization among different species of animals and microorganisms[1] stimulated our original experiments on patterns of purine utilization and turnover in the nucleic acids of different types of leukocytes in man. Until recently most of our work has been done with chronic leukemic patients who had enough cells for nucleic acid studies. We have begun to work with hematologically normal subjects, and we are using a modification of Kline's modification[7] of Ottesen's procedure[8] to separate normal lymphocytes and polymorphonuclear cells. We chose those patients with chronic lymphatic leukemia in whom the predominant cell type in the peripheral blood was the small lymphocyte. In patients with chronic granulocytic leukemia the leukocytes were polymorphonuclear. These patients had leukocyte counts of at least 100,000/cu mm, a reasonably good hemoglobin level, and were in good general condition so that they could be observed for a long period without specific therapy. Patients were given once intravenously one or two presumptive precursors of nucleic acids—either adenine-8-C^{14} or guanine-8-C^{14} alone, or one of these simultaneously with orotic acid-6-C^{14}. Blood was collected at intervals after the single injection of the labeled precursors. Leukocytes were separated from red cells by the use of dextran, and standard procedures were used to separate ribose nucleic acid (RNA) and deoxyribonucleic acid (DNA) from the leukocytes. RNA nucleotides were separated by paper electrophoresis, and the DNA bases or RNA purines only by paper chromatography. The radioactivities of the RNA nucleotides or purines and of the DNA purines and pyrimidines were measured

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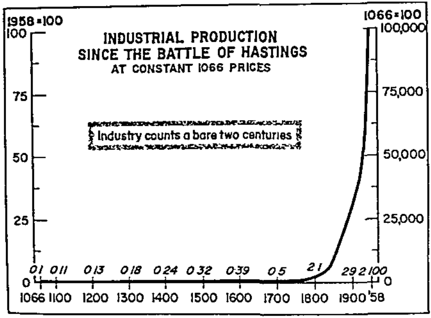


FIG 1—The conventional, simple minded view of what is known as the industrial "revolution (Figs 1 and 2 are reproduced by kind permission of *The Economist* London)

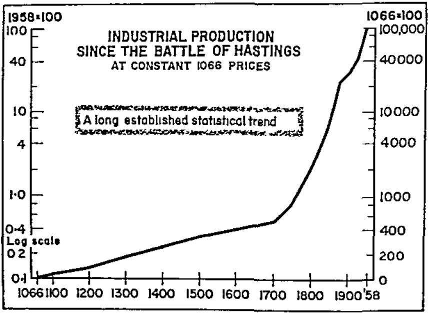


FIG 2—But the picture is quite false To compare rates of growth at different times one must go logarithmic, properly equating the doubling of industry s output between 1935–1958 with the corresponding proportionate rise in the number of copper saucepans in the middle ages

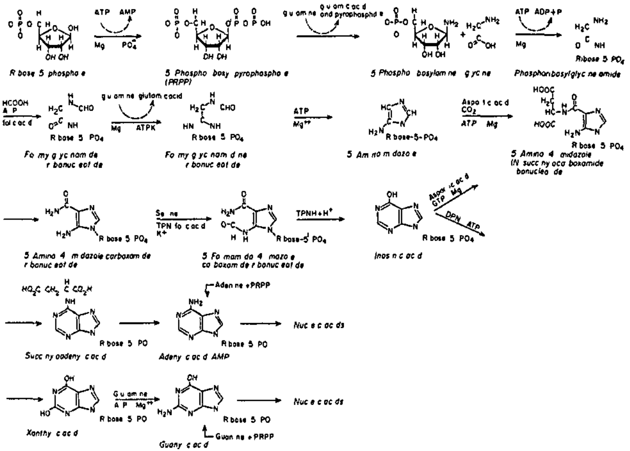
in an internal Geiger Muller flow counter (helium isobutane mixture) We have also studied extensively the incorporation of C^{14} formate into a variety of leukemic leukocytes

ADVANTAGES OF C^{14}

Use of carbon labeled precursors to label the DNA of hematopoietic cells has certain advantages over use of P^{32} (a) one is more likely to be labeling a constituent of DNA—one of the nitrogen bases—and therefore, not following the turnover of some rapidly metabolizing P compound that might be closely associated with the DNA (b) If the incorporation of the C^{14} precursor into RNA is studied at the same time, it provides a built in control of possible contamination of DNA with RNA, this could not be as readily controlled when P^{32} is used where there is a chance of metabolically active RNA contaminating the DNA and therefore giving a spurious DNA turnover This objection to P^{32} can of course be overcome by isolating the DNA labeled with P^{32} taking care to remove in this procedure any adherent RNA But, even with isolated DNA there still remains the small possibility of unknown adherent P compounds present in trace amounts, but with a high rate of turnover (c) The use of appropriate C^{14} labeled precursors makes it possible to label, efficiently and independently, all four nitrogen bases of DNA, and, therefore to follow simultaneously the turnover of a number of constituents in DNA This provides further detection of a possible metabolic heterogeneity of DNA and also additional information on the stability and interchangeability of labeled precursors (d) It is probable that P^{32} labels many P containing metabolites in the body some of which may persist and serve as a slowly released source of P^{32} this makes the interpretation of retention of P^{32} labeling in the DNA of hematopoietic tissues difficult to interpret. When C^{14} labeled precursors of nucleic acid are used they are specifically incorporated into the nucleic acids and their source in the acid soluble nucleotides is rapidly exhausted After a single intravenous injection in man the half time of a purine in the blood is ~90 minutes The availability of such specific C^{14} labeled precursors for nucleic acid synthesis is thus brief like an electrical impulse (e) It is possible that the terminal phosphates in DNA molecules might be labile and thus with an independent turnover, confuse over all turnover This difficulty would not arise with any C^{14} labeled precursor (f) It is much more difficult to detect impurities associated with a P compound whereas those arising from C^{14} labeled precursors of nucleic acids can be readily detected by ultraviolet spectrophotometry (g) Finally the longer half life of C^{14} makes it easier to follow retention of radioactivities over long periods of time With P^{32} this is not possible, and moreover correction has to be made for isotope decay

BIOSYNTHESIS OF NUCLEIC ACIDS

As previously reported,[5] figure 3 outlines the pathways of the synthesis of nucleic acid purines Since mammals do not require preformed nucleic acids, or any nucleic acid component other than the single atoms, the utilization of larger building blocks for nucleic acid synthesis, such as exogenous purines bypasses the normal synthetic pathway A wide variety of organisms utilize exogenous



Ribose 5 phosphate
ATP AMP
Mg PO₄
5 Phosphoribosyl pyrophosphate (PRPP)
glutamine glutamic acid and pyrophosphate
5 Phosphoribosylamine glycine
ATP ADP+P
Mg
Phosphoribosylglycineamide
HCOOH ATP folic acid
Formyl glycinamide ribonucleotide
glutamine glutamic acid
Mg ATPK
Formyl glycinamidine ribonucleotide
ATP Mg⁺⁺
5 Aminoimidazole
Aspartic acid CO₂ ATP Mg
5 Amino 4 imidazole (N succinylocarboxamide ribonucleotide)
5 Amino 4 imidazole carboxamide ribonucleotide
Serine TPN folic acid K⁺
5 Formamido 4 imidazole carboxamide ribonucleotide
TPNH+H⁺
Inosinic acid
Aspartic acid GTP Mg
DPN ATP
Succinyladenylic acid
Adenine +PRPP
Adenylic acid AMP
Nucleic acids
Xanthylic acid
Glutamine ATP Mg⁺⁺
Guanylic acid
Guanine +PRPP
Nucleic acids

FIG 3—1 Pathways in the biosynthesis of RNA and DNA purines

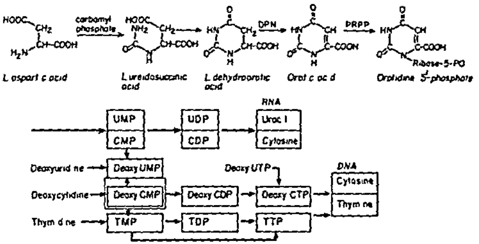


FIG 4—Pathways in the biosynthesis of RNA and DNA pyrimidines U uridine C cytidine, T thymidine, MP monophosphate DP diphosphate TP triphosphate

purines for the synthesis of polynucleotides, with much diversity in patterns of utilization [1] In man, exogenous purines are utilized for polynucleotide synthesis in both RNA and DNA Adenine appears not only as polynucleotide adenine but some is converted into polynucleotide guanine, similarly, guanine is utilized for both RNA and DNA guanine and is converted into polynucleotide adenine [3, 4] Thus, the injection of a single C^{14} labeled purine labels both DNA purines—adenine labels DNA adenine more than it does DNA guanine similarly guanine labels DNA guanine preferentially In no instance have we found any labeling of RNA or DNA pyrimidines after giving labeled purines

Figure 4 shows a similar flow diagram for the synthesis of nucleic acid pyrimidines Orotic acid is utilized in man as in other mammals for the synthesis of polynucleotide cytosine and uracil in RNA, and polynucleotide cytosine and thymine in DNA [5] C^{14} formate in man labels the purines in both RNA and DNA thymine in DNA, and, to a lesser extent, the other pyrimidines in both RNA and DNA. C^{14} thymidine labels, for all practical purposes, only the thymine in DNA

LABELING OF DNA PURINES AND PYRIMIDINES

Figure 5 charts the peripheral white counts of representative patients with chronic granulocytic and chronic lymphatic leukemia who received single injections of C^{14} labeled precursors Despite wide fluctuations in the peripheral count, the decline in radioactivities of the various components of the nucleic acids isolated seemed to follow a steady course and did not reflect these fluctuations. These results suggest that the white cells in the peripheral blood at any one time represent a small proportion of that particular cell type in the body, that these cells are a random sample of the total leukocytes of the body, and that radioactive labeling of the nucleic acids of leukocytes becomes relatively rapidly homogeneous for a given cell type, e g , the labeling of the nucleic acids in lymphatic tissue must equilibrate with the labeling of peripheral lymphocytes, as will be seen later from additional data

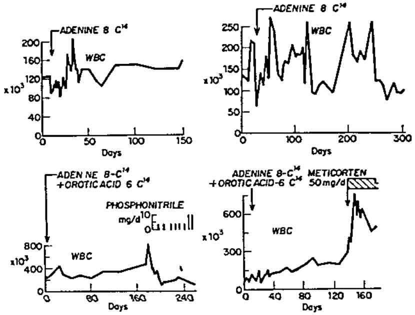


FIG 5 —Peripheral leukocyte counts and treatment of (a) M W ♂ age 30 with chronic granulocytic leukemia (b) F R ♂ age 47 with chronic lymphatic leukemia (c) C K ♂ age 66 with chronic lymphatic leukemia (d) J B ♂ age 48 with chronic lymphatic leukemia

Figure 6 shows the incorporation of adenine into the RNA and DNA of a patient with chronic granulocytic leukemia Turnovers of DNA adenine and guanine are very similar to those in RNA, suggesting that these cells are rapidly dividing A similar relationship holds in other rapidly dividing tissues e g , bone marrow and intestine Little radioactivity is demonstrable after 100 days In contrast in the lymphocytes of a patient with chronic lymphatic leukemia (fig 7) incorporation of adenine into the DNA is much less than that into the RNA, and besides there is an unexpected prolonged retention of radioactivity in both RNA and DNA purines the declines being parallel in both purines beginning at the point where both are equally labeled (D L Kline[8] has found a periodic peaking in radioactivity in the DNA of rabbit lymphocytes and lymph nodes for a short time after P^{32} Similar but less extensive fluctuations are found in parallel in the radioactivities of DNA adenine and guanine of chronic lymphatic leukemic leukocytes in the early part of the incorporation curves after adenine-8-C^{14} although our curves are drawn smoothly through these points) Similar results obtained when guanine was the precursor given to another patient with chronic lymphatic leukemia (fig 8) The lesser incorporation into DNA as compared with RNA is similar to the relationship of DNA to RNA in slowly dividing tissues such as liver

Figure 5d gives the main hematologic findings in a patient with chronic lymphatic leukemia who had received both C^{14} labeled adenine and orotic acid On day 127 after the C^{14} labeled bases he was treated with prednisone, an adrenocortical steroid It rapidly shrank the massively enlarged lymph nodes and spleen, and the white count rose from 200,000 to 800,000/cu mm , this increase presumably reflected the release of lymphocytes from shrunken nodes. During an 18-day period this patient was on a complete balance study He lost 3 4 Kg in weight, of which, from N and P ratios, ~1 8 Kg represented lymphatic tissue Despite this sudden large loss of lymphatic tissue and tremendous increase in the peripheral lymphocyte count, there was no change in the radioactivities of the DNA bases, which indicated the entirely uniform labeling of the lymphatic tissue of the body at this time Again, much less adenine was incorporated into the DNA of lymphocyte than into the RNA, but utilization of orotic acid for RNA nucleotides was not correspondingly in excess of that for DNA nucleotides (fig 9) Here, where all four nitrogen bases were labeled—DNA adenine, guanine, thymine, and cytosine and RNA adenylic, guanylic, uridylic, and cytidylic acids—there was prolonged parallel retention of radioactivity in all four bases

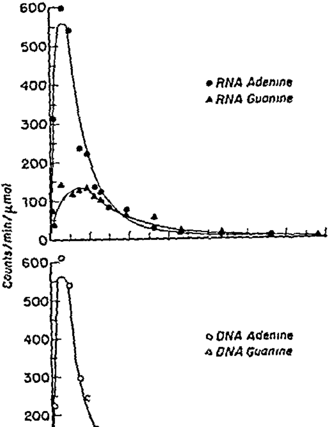
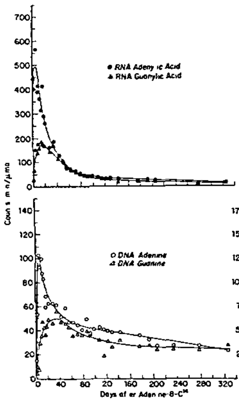


Fig 6 —Incorporation of adenine-8 C^{14} into RNA and DNA of granulocytes of M W *with chronic granulocytic leukemia*



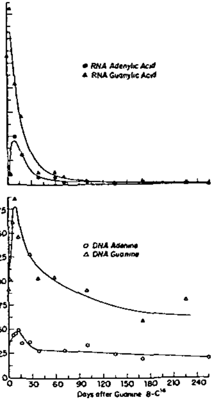


Fig 7 (*left*)—Incorporation of adenine 8 C^{14} into RNA and DNA of lymphocytes of F R with chronic lymphatic leukemia

Fig 8 (*right*)—Incorporation of guanine-8 C^{14} into RNA and DNA of lymphocytes of S B with chronic lymphatic leukemia

(both RNA and DNA) the decline in radioactivity proceeding in two phases an initial more rapid component and a residual slow component Treatment with prednisone did not affect the rate of decline of the four bases in DNA and RNA Similar data (fig 10) were obtained for another patient with chronic lymphatic leukemia who received C^{14} labeled adenine and orotic acid again treatment with diaziridinyl phosphonitrile—a triethylene melamine-like compound—which was associated with a substantial drop in the peripheral white count (fig 9c), did not affect the slow declines in retained radioactivities in the RNA and DNA nitrogen bases, as far as could be judged from the few succeeding values obtained after the drug was given

The analogous nucleic acid turnover pattern for the granulocyte—RNA and DNA turnover seeming almost identical—was obtained in another patient with chronic granulocytic leukemia who was given C^{14} labeled guanine and orotic acid The turnovers of RNA guanylic adenylic, uridylic, and cytidylic acids were very like those of DNA guanine, adenine, thymine and cytosine, respectively (fig 11)

The incorporation of adenine into the RNA and DNA of polymorphonuclear leukocytes from a patient with a leukemoid reaction (secondary to the metastases

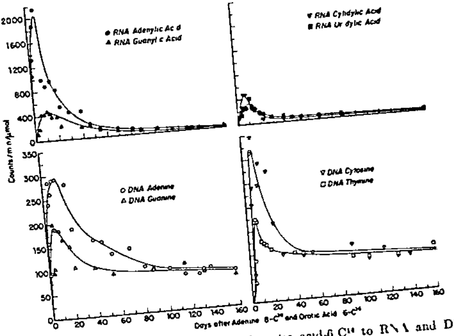


FIG. 9.—Incorporation of adenine-8-C^{14} and orotic acid-6-C^{14} to RNA and DNA of lymphocytes of J. B. with chronic lymphatic leukemia.

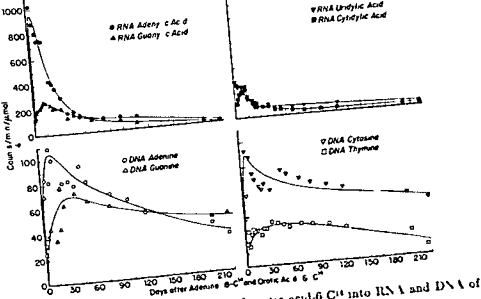


FIG. 10.—Incorporation of adenine-8-C^{14} and orotic acid-6-C^{14} into RNA and DNA of lymphocytes of L. K. with chronic lymphatic leukemia.

from carcinoma of pancreas) is given in figure 12. As in the granulocytes of patients with chronic granulocytic leukemia, adenine was rapidly and extensively incorporated into DNA adenine and guanine and similarly into RNA adenylic and guanylic acids. In contrast, the declines of radioactivity in the DNA purines

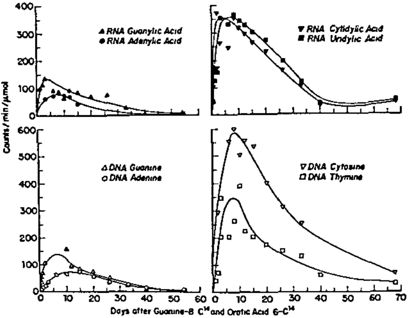


FIG 11 —Incorporation of guanine-8 C^{14} and orotic acid 6 C^{14} into RNA and DNA of granulocytes of A P with chronic granulocytic leukemia

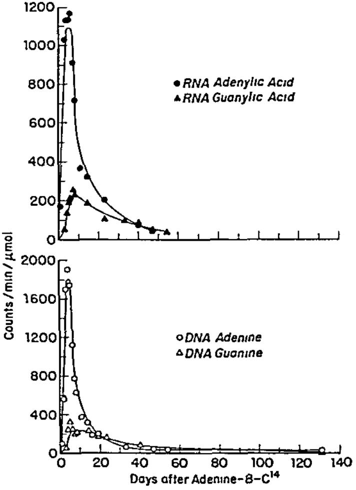


FIG 12 —Incorporation of adenine-8 C^{14} into RNA and DNA of polymorphonuclear leukocytes of G B with leukemoid reaction

and RNA nucleotides of polymorphonuclear leukocytes were more rapid than in the polynucleotides of leukemic granulocytes As suggested previously,[4] the more rapid turnover of DNA in the polymorphonuclear leukocytes indicate that these cells may have a shorter average survival time than their corresponding leukemic counterpart (at least in the chronic phase of the disease) but the apparent slower turnover of the DNA in the granulocytes of chronic granulocytic leukemia may have another explanation that will be discussed later

Preliminary results have been obtained on a hematologically normal 58 year old female patient with carcinoma of tongue and metastases to cervical nodes that had been resected She was given both C^{14} labeled adenine and orotic acid The results indicate that adenine as such is extensively incorporated into RNA and also transformed into RNA guanine in both normal polymorphonuclear leukocytes and lymphocytes Similarly the extent of incorporation and transformation of adenine into the DNA of polymorphonuclears is equivalent to the labeling found in RNA and exceeds that into the DNA of lymphocytes C^{14} orotic acid is likewise utilized by both normal lymphocytes and granulocytes for the synthesis of RNA cytosine and uracil and DNA cytosine and thymine Clearly utilization of exogenous purines and orotic acid for polynucleotide synthesis is not a unique property of leukemic or leukemoid leukocytes normal hematopoietic tissues also utilize them

An unusual feature of the labeling seen so far in the DNA of the normal polymorphs and lymphocytes is that the appearance of radioactivity in the DNA of lymphocytes precedes by several days labeling in the DNA of polymorphs the latter being delayed from four to six days before it begins to increase rapidly This finding we have not previously made after C^{14} purines and orotic acid were given to patients either with chronic granulocytic leukemia or with leukemoid reactions In the latter two instances there was no delay in the appearance of label in the DNA of the peripheral granulocytes this activity has increased rapidly Our present results on the appearance of label in normal polymorphonuclears resemble results that have been described by Ottesen[11] (cf fig 13) and by Kline[8] after P^{32}

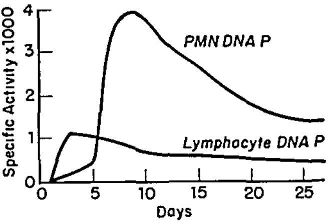


FIG 13 —Incorporation of P^{32} into DNA of polymorphonuclear leukocytes and lymphocytes (Reproduced by permission of J Ottesen and editor Acta physiol scandinav)

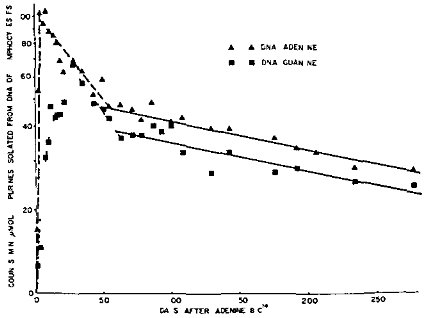


FIG 14 Incorporation of adenine 8-C^{14} into DNA of lymphocytes of F S with chronic lymphatic leukemia (Reprinted from Brookhaven Symposia in Biology 10 1957 [5])

phonuclear cells are apparently being released more rapidly from the bone marrow than normally in the normal there is a delay of >4 days before labeled polymorphonuclears appear in the peripheral blood This delay probably represents a combined storage or maturation time in the bone marrow after the last division in granulocyte development

SURVIVAL TIME OF LEUKOCYTES

We have previously discussed our misgivings on the use of these DNA turnover data to fix the survival time of leukocytes[4, 5] The first assumption that

TABLE 1.—*Incorporation of Adenine-8-C^{14} into RNA and DNA of Chronic Granulocytic Leukemic Leukocytes, Leukemoid Polymorphonuclear Leukocytes, Normal Polymorphonuclear Leukocytes and Lymphocytes (Radioactivity of Isolated Nucleotide or Purine in counts/min/µM)*

	Time after Adenine-8-C^{14} 200 µc.		30 min	60 min	120 min	300 min	24 hrs	48 hrs.
D B ♂ 66 chronic granulocytic leukemia	RNA	Adenylic	188	319	371	403	456	822
		Guanylic	—	—	—	25	84	100
	DNA	Adenine	—	25	45	63	238	595
		Guanine	—	—	—	—	—	65
G B ♂ 58 leukemoid reaction Polymorphs	RNA	Adenylic	—	23	55	65	393	1257
		Guanylic	—	—	—	—	—	41
	DNA	Adenine	—	—	7	12	103	561
		Guanine	—	—	—	—	—	31
								4 days
I McG ♀ 58 Polymorphs	DNA	Adenine					—	—
		Guanine					—	—
Lymphocytes	DNA	Adenine					127	316
		Guanine					—	—

A blank space in the table indicates sample not measured, a dash in the table indicates no significant radioactivity.

Note. Although the over all turnover of RNA and DNA are very similar in chronic granulocytic leukemic leukocytes and leukemoid polymorphonuclear leukocytes, *incorporation into RNA significantly precedes incorporation into DNA.*

DNA once formed in a cell is metabolically stable throughout the life of that cell, seems valid enough. There is no clear evidence of metabolic turnover of DNA independent of cellular division, apart from some aberrant and rare radioautographic observations. But do leukocytes have a finite life like the life span of erythrocytes—or does their probability of destruction change very little with age? If we assume that there is no turnover of once-formed DNA in a cell, then the inconspicuous plateaus in our data suggest a random disappearance of leukocytes from the circulation, not finite survival as with erythrocytes. But plateaus would not be conspicuous in short lived cells. Although these reservations lead one to question the meaningfulness of average survival times of leukocytes derived from DNA turnover studies, we have calculated these (from the Volterra integral equation of the first kind, as used by Shemin and Rittenberg[14]) from our DNA data. As we have defined our use of the term previously,[4, 5] it should again be emphasized that average survival time from our data includes the time from the earliest formation of the cell in the bone marrow to its last appearance in the peripheral blood. It thus includes all the time spent in division and maturation in marrow or lymph nodes, and the time spent outside the vascular bed in any state before release into the circulation, as well as the actual time spent intravascularly. If some leukocytes spend their time recirculating, it would include also the time until their final disappearance extravascularly. Again, it must be emphasized that the average survival times represent the average of cells surviving for a shorter period and others surviving longer, and that the

TABLE 2 — *Average Survival Time of Leukocytes in Man*

Cell Type	Author	Method	Average Survival Time in Days
Leukocytes (all forms)	Kline and Clifton[6]	DNA P^{32}	13
	Weisberger and Levine[15]	L cystine S^{35}	13
Normal Polymorphonuclear Leukocytes	Ottesen[11]	DNA P^{32}	9
	Hamilton[4, 5]	DNA C^{14}	9
Chronic Granulocytic Leukemic Leukocytes	Osgood et al [9]	DNA P^{32}	3
	Rigas[13]	DNA P^{32}	4
	Weisberger and Levine[15]	L-cystine S^{35}	13
	Hamilton[4, 5]	DNA C^{14}	23
Normal Lymphocytes	Ottesen[11]	DNA P^{32}	(a) 3–4 (b) 100–200
Chronic Lymphatic Leukemic Leukocytes	Osgood et al [9]	DNA P^{32}	30
	Osgood et al [10]	DNA P^{32}	84
	Weisberger and Levine	L-cystine S^{35}	prolonged
	Hamilton[5]	DNA C^{14}	30–85

Note E P Cronkite and his associates at this conference reported an average survival time of nine days using DNA Thymidine H^3 labeling

range from our data is considerable[4] Table 2 summarizes such analyses carried out by others and ourselves

THE REUTILIZATION HYPOTHESIS

What is the significance of the prolonged retention of radioactivity in lymphatic tissue? If radioactivity in the DNA of the lymphocyte is plotted against time (fig 14) the decline in radioactivity falls into two phases an initial rapid decline then a slow decline It is conceivable that the slow decline (with a half time of nearly a year) represents a second type of leukemic lymphocyte with a much longer life than 85 days Or might this represent the reutilization by lymphocytes of large fragments of the nucleic acids or nucleoproteins of their progenitors? Alternatively as E P Cronkite has suggested this might represent the slow release of labeled cells derived from primitive precursor cells labeled at the time the isotope was originally given This latter explanation would not be inconsistent with our failure to find higher labeling in lymph nodes or to perturb the rate of decline in radioactivities in the DNA purines by altering total lymph node mass We have already discussed extensively the evidence that led us to wonder about reutilization of whole lymphocytes The recent work of Resegotti[12] who found the same biphasic decline in radioactivity in the hemin iron of lymphocytes lymph nodes, and thymus of rabbits after labeling with Fe^{59} would be consistent with the postulated reutilization Similarly, we have reviewed morphologic data[5] that speak in favor of such a reutilization and reason for our attraction to this highly speculative hypothesis Although there has been no new evidence to support this hypothesis and the simpler explanation would be the persistence of some lymphocytes for very long times, the possibility of such reutilization must be kept in mind as perhaps applying to all stages of lymphocyte production.

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Homologous and Heterologous Transplantation of Radioactively Labeled Lymphocytes as a Method of Studying Their Function

By KARL-ERIK FICHTELIUS

DURING THE PRESENT DECADE the old question of the fate of lymphocytes following their disappearance from the blood has been reinvestigated by means of transplantation[8, 10-16, 2, 33, 39]* The present paper discusses how well this method is suited to the problem, especially in light of results obtained in experiments with radioactively labeled lymphocytes.

The same laws apply to the transplantation of lymphocyte suspensions from one animal to another as apply to transplantation of all tissues. The graft is rejected as the result of an antigen-antibody reaction after antibodies have been formed against it.[23] Tissue antibodies have not been observed before the third day after transplantation.[4] The difference between hetero- and homotransplantation is one of degree, the heterologous cell having a greater antigenic effect. When the transplantation takes place between siblings (syngenesiotransplantation) the effect is less than in homotransplantation. Under certain conditions where the formation of antibodies in the recipient is inhibited, heterologous grafts can survive for a protracted period.[3, 9, 17]

During the first phase of immunization, some of the antigen is taken up in the reticuloendothelial system (RES). The greater part is destroyed much later, in most cases after a week, by means of the antigen-antibody reaction.[9] It is theoretically possible that most of the transplanted lymphocytes can function in one way or another before they succumb to an antigen-antibody reaction on the third day or later. The great number of investigations concerning the passive transmission of hypersensitivity of the delayed type and the capacity to form antibodies with living lymphocytes gives strong support to the hypothesis that the lymphocytes function in the recipient. Bibliographies of works on this subject have been compiled by Wesslen,[40] Skog[35] and Stavitsky[36] among others. Harris and Harris[23] compared the importance of antibody formation against the transplanted lymphocytes to that of antibody formation by the transplanted cells themselves. Previously irradiated recipients of lymph node cells from immunized donors showed higher serum concentrations of agglutinins than normal recipients of similar suspensions; this difference is first demonstrable on the third day. This is in good agreement with Bollag's observation[4] of tissue antibodies on the third day. In an experiment of the same type, transplanted cells were taken from nonimmunized donors and incubated in vitro together with antigen. A similar decrease of antibody formation in normal recipients as compared to irradiated ones was noted as late as the fifth day after transplantation.

A possibility that must be taken into account, however, is that transplanted

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* The word "transplantation" is used to designate transfusion as well as transplantation.—*Ed.*

lymphocytes may be destroyed by antibodies present in the recipient at the moment of transplantation Such a mechanism is uncommon and cannot affect conclusions based on experiments comprising a series of transplantations[4]

After this introductory survey of the biologic background of lymphocyte transplantation, we turn to a discussion of the possible ways in which the transplanted cells may be dealt with by the recipient Our arguments are based on results obtained with lymphocytes labeled with P^{32}

1 DISSOLUTION OF THE TRANSPLANTED LYMPHOCYTES IN THE BLOOD

Some of the transplanted cells may be destroyed in the blood stream, but the results so far obtained exclude the possibility of all being destroyed within 24 hours[11] Thymus cells labeled with P^{32} were transplanted from one rat to a litter mate After 24 hours, the distribution of P^{32} in several different organs was investigated and compared with the distribution following an injection of inorganic P^{32} It was found that after transplantation of thymus cells, the relative activity in the liver and spleen was greater than after injection of inorganic phosphorus If all the cells had been completely destroyed after 24 hours, their P^{32} ought to have been distributed in the same way as inorganic phosphorus injected directly into the blood This conclusion presupposes that the control experiment with inorganic P^{32} is reliable

Two kinds of control have been used when transplanting lymphocytes labeled with P^{32} injections of labeled but destroyed lymphocytes and injections of inorganic P^{32} Both types have been compared by Weisberger et al[33] The distribution of radioactivity among lung, liver, spleen, and kidney was the same 10 minutes after intravenous injection of inorganic P^{32} or lymphocytes labeled with P^{32} and destroyed by supersonic vibration This shows that the destroyed cells, which had probably been taken up by RES, very quickly released all phosphorus, including that bound as nucleoprotein

Research into the behavior of the lymphocyte requires experiments of longer duration[32] For primary transplantation to unprepared recipients, one to three days is a suitable period (see above) We know from Weisberger's experiments that the phosphorus in the destroyed cells is quickly released and distributed like inorganic P^{32} and that the control with P^{32} suffices for a test period of 10 minutes But what is the situation when the experimental period is longer? Can the transplanted cells, some of which are damaged even when carefully handled, influence the recipient in such a way that free P^{32} is distributed differently than when inorganic P^{32} is injected? In my experiments the distribution of DNA P^{32} has been determined and this is dependent mainly on the mitotic frequency of the organs The question now arises of whether a substance in the suspension of transplanted cells can cause the mitotic frequency to increase in these organs to which lymphocyte activity could be traced The activity of the transplanted thymus cells was traced to the liver and the spleen It is most improbable that an injection of cells can cause the mitotic frequency of the liver to increase The higher relative activity in the liver must therefore be due to P^{32}, still bound in some way or other to whole lymphocytes The experiment reported shows that not all cells were destroyed after 24 hours.

In similar investigations, Fichtelius[15] showed that not all the cells have been destroyed 48 hours after transplantation. Osogoe[31] claims to have found unlabeled transplanted lymphocytes in the liver and spleen 72 hours after transplantation. Harris and Harris[23] show that transplanted lymphocytes very probably produce antibodies as late as the fifth day after transfusion to untreated recipients.

Injection of inorganic P^{32} as a control may be criticized because the injected cell suspension may affect the recipient in such a way that the liberated P^{32} is distributed differently than when inorganic P^{32} is injected. Such a mechanism cannot explain my results, but it is not impossible that they are influenced by it. A comparison between an injection of inorganic P^{32} and one of nonviable cells over a longer experimental period than Weisberger's does not add much, since the control experiment with destroyed cells is far from ideal. It is not at all certain that destroyed cells affect the recipient in the same way as predominantly intact and motile cells. Control experiments consisting of an injection of inorganic P^{32} are very much simpler than those with destroyed labeled cells. Under the present conditions it seems most sensible to use injections of inorganic P^{32} in control experiments, in each case bearing in mind the possibility of an influence of the suspension on the recipient.

2 Phagocytosis of the Transplanted Lymphocytes in RES

As mentioned above, the activity of rat thymus lymphocytes transplanted to the rat was traced to the liver and spleen. The activity of similarly transplanted rat lymph node lymphocytes was present in liver and bone marrow, and probably also in lymph nodes and lung.[14] The activity of rabbit lymphocytes from both thymus and lymph nodes transplanted to rats was traced to the liver.[14] These results cannot be explained by simple phagocytosis of the transplanted cells in the RES. If phagocytosis of RES cells is responsible for the distribution of the transplanted cells in the recipient, the activity of these cells should in all cases be apparent in the RES organs, namely, the liver, bone marrow and spleen. It may be argued that the distribution of phagocytic particles among the different RES organs depends on the size of the particles. Gabrieli,[18] who studied the RES by means of $Cr^{51}PO_4$, found that when the injected particles were coarse, the lungs absorbed the major part of the colloid, whereas if the dispersion was finer, the liver took up more. In Gabrieli's experiments the spleen absorbed equal amounts of the coarse and the fine dispersion. Even this knowledge about the RES function cannot explain the results with lymphocytes labeled with P^{32}. Why are heterotransplanted lymphocytes traced only to the liver, while syngenesiotransplanted ones are recovered in other RES organs as well? Why can syngenesiotransplanted lymph node cells be found in the bone marrow but not in the spleen, while thymus cells transplanted in the same way appear in the spleen but not in the bone marrow? This difference between syngenesiotransplanted thymus cells and lymph node cells shows that there is more to the matter than a simple phagocytosis of the transplanted cells in RES.

3 Specific Reutilization by Lymphocytes of Large Fragments of Nucleic Acid of Their Progenitors

In order to explain the long life span of the lymphocytes in experiments based on their DNA metabolism,[5, 10, 21, 30, 34] Hamilton[20, 21] advanced a hypothesis suggesting a specific re-utilization by lymphocytes of large fragments of nucleic acid or nucleoproteins. This theory was morphologically tested by Trowell,[47] who started from the well known fact that many small lymphocytes are phagocytosed by reticular cells in the reaction centers of lymphoid tissue.[77] When culturing lymph node cells in vitro, he found that cells, which had phagocytosed small lymphocytes, were differentiating into large lymphocytes. According to him this provides a histologic basis for the re-utilization hypothesis.

It is not my intention to go into all the details of this theory. What is important is to find out whether it provides an explanation for the results obtained from transplantations of lymphocytes labeled with P^{32}. According to the hypothesis, the lymphocytes are phagocytosed by the RES, and $DNAP^{32}$ is re-utilized in that part of this system where lymphocytes are formed—in my experiments the lymph nodes, thymus, and spleen. Thus, it should be possible to trace $DNAP^{32}$ for a limited period and to make a comparison with the injection of inorganic P^{32}. In the remaining RES organs—the liver, lungs, and bone marrow—$DNAP^{32}$ is thought not to be re-utilized and cannot be recovered after the above interval. In my experiments,[11, 14] where the recipient was examined after 24 hours, no activity from the transplanted lymphocytes should be found in the liver, lung, and bone marrow. This is not the case, however, for in all my experiments part of the activity of the transplanted cells could be found in the liver. In syngenesiotransplantation of lymph node lymphocytes, the labeled cells were found in the bone marrow as well. Even if we presuppose that the DNA of the phagocytosed lymphocytes can be re-utilized for the formation of other cells, my results cannot be explained by phagocytosis of the transplanted lymphocytes and re-utilization of their DNA. This is evident from the fact that in syngenesiotransplantation of lymph node cells and in heterotransplantation of lymph node and thymus cells, no activity of the transplanted cells could be discovered in the spleen, where the phagocytosis of lymphocytes is lively.[74]

4. Filtration of the Transplanted Lymphocytes in Liver and Spleen

In investigations concerning the destruction of red cells, Jandl et al.[46] showed that the spleen, liver, and lung function as passive filters with differently sized pores. Small agglutinates are caught mainly in the spleen, while larger ones reach the liver and lung. Labeled cells caught in a filter are not necessarily broken down. How well can such a mechanism explain the results of transplantation of labeled lymphocytes? The mechanism described by Jandl et al. presupposes agglutination of the transfused cells. Such agglutination does not occur to any great extent in transplantation from rat to rat and from rabbit to rat.[8] My results,[11, 14] therefore, can hardly be explained in this way. Moreover, it is impossible by such a mechanism to explain the results reported below.

5. Factors Supporting the Theory That Transplanted Lymphocytes Function Within the Recipient

None of the above mechanisms alone or in combination, can explain the results obtained from transplantations of lymphocytes labeled with P^{32}. Another possible explanation for these results is provided by the supposition that the transplanted cells survive and function in one way or another within the recipient. Some arguments in favor of such a hypothesis follow below.

A In the experiments where thymus cells labeled with P^{32} were transplanted from rat to rat and from rabbit to rat,[13] peritonitis was induced in the recipient by intraperitoneal injection of liquid paraffin two days before the transplantation Twenty four hours after the transplantation, the animals were killed, and the distribution of radioactive phosphorus among certain reference organs, as well as in the peritoneal exudate cells, was determined It appeared that the relative activity in the exudate cells was higher after heterotransplantation than after syngenesiotransplantation The relative activity in the exudate cells was also higher after heterotransplantation than after injection of inorganic phosphorus This is interpreted as evidence that the heterotransplanted cells form exudate cells in the recipient [13] It is surprising to find that it is the heterotransfused cells and not the syngenesiotransfused ones that form exudate cells under the prevailing conditions The difference between the behavior of these two kinds of cell could be due to the different immunologic states of the donors This hypothesis is discussed in more detail elsewhere[13] and is supported by the following argument in favor of the functioning of transplanted cells within the recipient

B Thymus and lymph node cells labeled with P^{32} were transplanted from rabbit to rat [16] In one series, both donor and recipient were immunized with *B typhosa* H antigen, and in another series, only the recipient and not the donor was immunized A comparison of the distribution of radioactivity among various organs 24 hours after transplantation revealed a clear difference between the two series Immunization of the donor somehow affects the lymphocytes, so that they are dealt with differently by the recipient [16] The experiment proves in an indirect way that the transplanted lymphocytes are concerned with the formation of antibodies, and strongly supports the hypothesis that the transplanted cells function in the recipient

C Most histologists agree that there are no significant differences between lymphocytes in the thymus and those in the lymph nodes [2, 7, 22, 41] Uncertainty, however, has arisen as to whether thymocytes can indeed be lymphocytes, since the thymus sometimes reacts differently than lymphatic tissues [1, 24, 38, 42] Lymphocytes of both types labeled with radioactive phosphorus were injected intravenously from rat to rat [14] There was a distinct difference in behavior between the two types of cell, the thymus cells being traced to the liver and spleen and the lymph node cells to the liver and bone marrow The most plausible explanation for this difference is that the transplanted cells fulfill some function in the recipient

Summary

The old question concerning the disappearance of the lymphocytes from the blood has been investigated by many workers with the aid of transplantation of lymphocytes from one animal to another The suitability of this method to the problem is discussed on the basis of experiments with radioactively labeled lymphocytes The following conclusions are reached

1 Not all transfused lymphocytes are dissolved in the recipient during the experimental period under discussion, one to three days

2 The results of transfusion of labeled lymphocytes cannot be explained by a simple phagocytosis of the transfused cells in RES

3 The results cannot be explained by specific re-utilization by lymphocytes of large fragments of nucleic acid

4 A simple filter function of the organs in which transfused lymphocytes are recovered does not satisfactorily explain the results

5 Instead, the results obtained support the hypothesis that transfused lymphocytes can function in the recipient

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On DNA Labeling in the Study of the Dynamics of Bone Marrow Cell Populations

By L. G. Lajtha

IT IS GENERALLY ACCEPTED that in most growing tissues DNA is stable, that is, net synthesis may occur, but no significant turnover. Consequently, in a population of cells with complex dynamics, such as the marrow cells, labeling of DNA and following the fate of this label promised a method for the study of the processes involved. A great deal of work has been done on these lines, the two ways in which the fate of the label was followed being specific radioactivity of a compound (DNA or its components isolated) or direct investigations of cells with high resolution autoradiography.

Recently the metabolic stability of DNA—the basic assumption of experiments with DNA labeling—has been questioned by Pelc.[8] While he does not claim general validity of his observations, they are of sufficient importance to warrant investigations of specific tissues. However, for the arguments to be presented, it is assumed that in bone marrow cells DNA is stable, and the purpose of this paper is to point out certain difficulties that render the interpretation of results even with stable DNA labeling somewhat uncertain.

Pitfalls of Specific Radioactivity

In any cell population, 100 per cent of the cells synthesizing at a given rate, or 25 per cent at 4 times that rate would give identical specific activities. Beside actual rate differences, DNA is synthesized only during part of the interphase (S period), and at any time a varying proportion of cells may be in their S period.[4] Furthermore, bone marrow is a very heterogeneous population of cells, apart from the main erythroid and myeloid series of cells there are different stages of maturation within each series—indeed, specific activity determinations can give only very meager information.

The situation is somewhat different when so-called pure cell lines can be isolated, such as lymphocytes from the thoracic duct or neutrophils from the peripheral blood. The rate of appearance of the label in the periphery may reflect the rate of production of mature cell forms (assuming that most of these do not synthesize DNA), and the rate of disappearance of the label in the periphery may indicate the life span of the cells investigated, provided that there is no significant reutilization of the label. It appears, however, that there is a significant reutilization of DNA breakdown products in the body. In a human adult there are about 10^{12} cells produced daily. In terms of purine base content per cell this means a daily utilization of about 2.8 Gm. of purine bases. The daily uric acid excretion is of the order of 0.5 Gm., the dietary intake about 0.25 Gm. —indicating a limited amount of purine neosynthesis ($^1/_{10}$ to $^1/_5$ of the daily requirement) and an efficient reutilization of about $^4/_5$ of the daily nucleic acid

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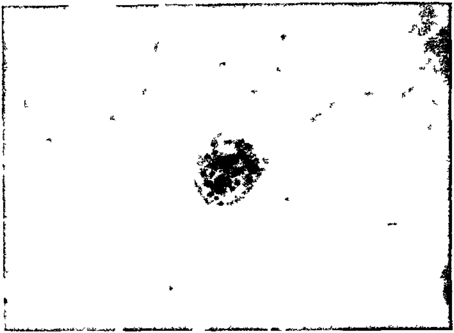

FIG 1 —Basophilic normoblast from human marrow cultured with 0 1 μc /ml medium H^3 thymidine (360 μc /μM) Stained autoradiograph × 3000

breakdown With different tissues having different cell turnover the interpretation of specific activity life span curves becomes very uncertain—except for the fastest growing tissue, if it is known which tissue this is

On theoretical consideration, the reutilization of DNA phosphorus may be less than that of purines because of the large body phosphate pools but it should be noted that (a) the same pools have compartments with different turnover times (b) the isolation of pure DNA phosphorus is difficult (c) P^{32} has a relatively short half life, thus necessitating larger doses of P^{32} than of C^{14} adenine and (d) even moderate amounts of P^{32} may deliver an appreciable dose of total body radiation possibly interfering with rates of proliferation

It appears from the arguments cited above that radiochemical measurement of DNA specific activities is of no use with bone marrow cells when attempting to follow cellular dynamics, and of very limited use even with relatively pure lines of cells

AUTORADIOGRAPHY

With the introduction of tritium labeled thymidine a highly efficient specific DNA label became available Tritium gives superb autoradiographs (fig 1) and even in closely packed cells (sections) there is no doubt as to which cell contains the label The amount of label per labeled cell (grain count/+ve cell) indicates the relative amount of DNA synthesized provided that the intracellular precursor pools are the same,* and provided that all cells have the same thickness

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* In vivo experiments in different animals or even in the same animal at different times also need to take the extracellular pools into consideration

(the average range of β particles from H^3 being only about 1μ). However, we possess no information on the intracellular precursor pools in different cells, nor a good idea of the thickness of nuclei of different cells; consequently the grain counts over individual cells must be interpreted with considerable caution.

This difficulty can be overcome by using C^{14} labeled compounds to check grain count differences. Firstly, the range of C^{14} electrons is much greater ($\sim 60\mu$) than cell thickness variations on smears (3 to 6μ), and secondly, the lower specific activity of C^{14} compounds necessitates the administration of larger amounts of labeled material, thus decreasing the influence of intracellular pools. In our laboratory, all thymidine H^3 grain count patterns are checked with C^{14} formate, which, in vitro, is nearly as specific a DNA label as is thymidine.[3]

The proportion of labeled cells (%+ve cells) in a population may indicate the relative length of the DNA synthetic period to that of the total cell cycle. If the length of the S period would be the same for all cells in the body, then the %+ve figure would give a good indication of the cell cycle (i.e., growth rate) of different tissues—provided the length of the S period has been measured (fig 2a). However, if the length of the S period varies or cells do not synthesize their full complement of DNA, serious miscalculations may result (fig 2b). As in most tissues so far investigated, the length of the S period seems to be of the order of 5 to 10 hours, the %+ve figures alone may give a *rough* indication of the growth rate of the cells in question. However, it is essential to establish that the cell population in question is homogeneous. For example, in a population of lymphocytes (human or rat, normal or leukemic), the %+ve figure at any time is about 2 to 3 per cent. The length of the S period being about the same as for other cells, this figure of 2 to 3%+ve would indicate a very long cell cycle indeed for the lymphocyte; if S = 10 hrs and %+ve = 2 per cent, then cycle time = 500 hrs. On closer investigation, however, it was noted that the lymphocyte population is far from being homogeneous; over 90 per cent of the cells are small lymphocytes (0%+ve) and usually 5 to 10 per cent of the cells are medium and large lymphocytes (30%+ve). In other words, the large lymphocytes have a cell cycle similar to young bone marrow cells (myelocytes, normoblasts, cycle time 20 to 30 hrs), while the small lymphocytes do not synthesize DNA. Naturally, changes of the over all %+ve figure over a lymphocyte population are meaningless, unless the proportion of large lymphocytes is known.

Even in a morphologically homogeneous population of cells, such as blast cells from leukemia, it is essential to determine whether the %+ve figure obtained after an instantaneous labeling represents a functionally homogenous population of cells, e.g., 10 per cent dividable cells with a short cell cycle or 100 per cent dividable cells with a long cell cycle. For such determinations either a continuous infusion of the label or a prolonged in vitro contact, preferably 10 to 20 hours, with the label is required to see if the %+ve figure increases with time, whether the increase follows a straight line and whether it ever reaches near 100 per cent. It should be noted that in a mixed population of young and differentiating cells, this determination can only refer to the young cells, since maturation of early labeled cells to late forms during the experimental period may create a false picture of increase in the proportion of labeled late forms.

The advantages of autoradiography are that complex cell population dynamics

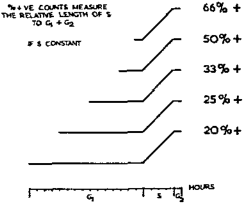

FIG 2A

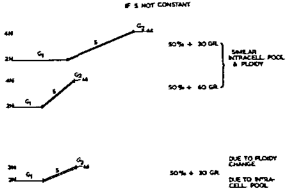

FIG 2B

FIGS 2A and B —Cell cycle measurements and growth rate In a homogeneous population with constant DNA synthetic period (S) the %+ve figure indicates the length of cycle However if S is not constant the same %+ve figure may be obtained even though the cycle times differ by a factor of 2 or more In such cases the grain counts over labeled cells may indicate the rate of synthesis assuming (a) even cell thickness (b) similar intracellular precursor pools (c) similar amount of DNA synthesized

For accurate measurements the following have to be determined

(1) %+ve figure (relative length of S to $G_1 + G_2$)
(2) its rate of increase with time up to 90 to 100 per cent (homogeneity in terms of capacity for DNA synthesis)
(3) time of continuous labeling needed to reach maximum grain counts (length of S period)
(4) grain count distribution over labeled cells } homogeneity in terms of ploidy
(5) DNA content per cell (distribution)

can be followed, and reutilization of DNA components are not a real problem because of an adequate dilution of the released label However it has been suggested that in lymphatic tissues bodily" reutilization of nuclei may occur [2, 11] In this case, of course, the label would not be diluted much and misleading information as to life span of the cells would be obtained

Another complicating factor in the interpretation of autoradiographic findings is the migration and morphologic transformation of cells In bone marrow or

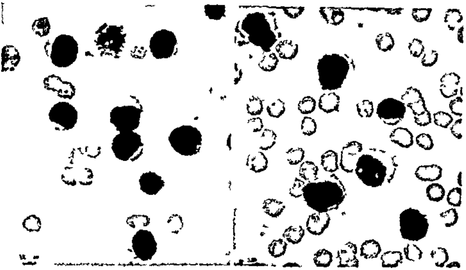

FIG 3A FIG 3B

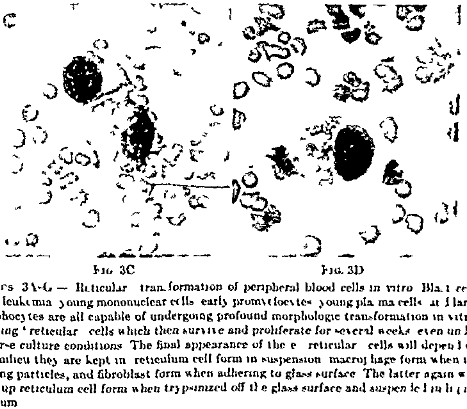

FIG. 3C FIG. 3D

FIGS 3A-D — Reticular transformation of peripheral blood cells in vitro Blast cells from leukemia young mononuclear cells early promyelocytes young plasma cells and large lymphocytes are all capable of undergoing profound morphologic transformation in vitro yielding "reticular" cells which then survive and proliferate for several weeks even under adverse culture conditions The final appearance of these "reticular" cells will depend on the milieu they are kept in reticulum cell form in suspension macrophage form when ingesting particles, and fibroblast form when adhering to glass surface The latter again will take up reticulum cell form when trypsinized off the glass surface and suspended in liquid medium

A Blast Cells peripheral blood—0 hours B Infectious mononucleosis peripheral blood
C Transitional forms after 48 to 72 hours culture D Macrophage in 120 hours culture

blood cell cultures, a very profound morphologic transformation may be observed after 5 to 10 days incubation the change of early cell types into "reticular" forms The cells may assume the appearance of reticulum cells, macrophages, or fibroblasts, depending on the mode of culture, and can grow more or less in-

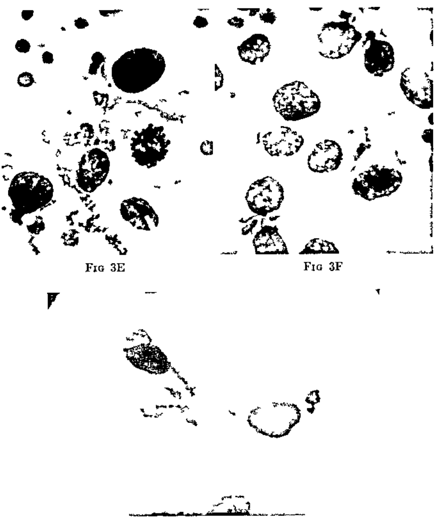

FIG 3E FIG 3F

FIG 3G

E Reticulum cells in 150 hours F Reticular syncytium in 150 hours culture G Fibroblast forms in 150 hours culture

definitely (fig 3) It has been suggested that such transformation may occur in vivo [9] and until its extent is established in any particular experiment it represents a potential source of error in interpretation

Even more complicated are the problems presented by differentiation Metabolic functions appear to proceed at a faster rate in young cell forms than in the mature forms Table 1 indicates the difference in rate of DNA synthesis between young and mature cell As grain count differences between different cells labeled with tritium might have been the result of differences in cell thickness the experiments were repeated with formate C^{14} The rate of DNA synthesis in the late forms per cell is about half of that in the young forms Similar findings are obtained with RNA label labeled amino acids with Fe^{59} in the erythroid series [7] and with S^{35} both in vitro and in vivo in the myeloid series [8] When the

TABLE 1.—*Rate of DNA and RNA Synthesis in Relation to Degree of Differentiation in Bone Marrow Cells*

(3 hours incubation in vitro grain/labeled cell)

	Basoph. normoblast	Polychr. normoblast	Promyelocyte	Myelocyte
Thymidine H^3 (DNA)	74	59	60	37
Formate C^{14} (DNA)	34	16	35	15
Adenine C^{14} (RNA)	60	19	50	26

Note The same principle is valid in respect to uptake of labeled amino acids, Fe^{59} or S^{35} sulfate

proportion of cells in DNA synthesis is investigated a similar difference is noted between early and late cell forms e g 66%+ve early basophilic normoblasts–33%+ve polychromatic normoblasts or 60%+ve promyelocytes–30%+ve myelocytes. In cycle times this would mean basophilic normoblast —— S = 12 hrs %+ = 66% —— cycle time 18 hrs polychromatic normoblast —— S = 20 hrs %+ = 30% —— cycle time 66 hrs However this implies that the polychromatic normoblasts synthesize the full 2n amount of DNA which may or may not be so, and also implies that the polychromatic normoblasts are a homogeneous population, which they are certainly not Even the most conscientious definition of polychromatic normoblasts includes cells at different stages of maturation As seen from figure 4 a proportion of cells which will be called middle polychromatic normoblasts will never synthesize DNA Consequently the cycle time as derived from the %+ figure in these cells is unduly long This situation is further complicated by the fact that an appreciable portion of early cell forms (e g , basophilic normoblasts) can mature into reticulocytes without intervening cell division[10] (fig 4) thus introducing a disturbing influence on any grain count distribution or %+ figure over the later cell forms

Interestingly enough the leukemic blast cells although presumed to be the "youngest" forms, show a slower rate of DNA synthesis and longer cell cycle than normal myelocytes or normoblasts (table 2) Their RNA or protein synthesis is also slower than that in myelocytes and normoblasts, and is somewhere between these cells and the very slightly active lymphocytes (table 3) The paucity of normal blast cells in normal marrows makes statistical comparisons between normal and leukemic blast cells difficult but it should be noted that no difference of this kind is found between other identical cell types (e g myelocytes or lymphocytes), whether normal or leukemic

Another source of error lies in the inaccuracy of marrow differential counting Those experienced in marrow cytology are only too well aware of the not negligible proportion of cells labeled as unclassified The more accurately one attempts to classify marrow cells, the higher is the proportion of these cells On autoradiographs, the staining of the cells is never as good as on a plain smear, an occasional heavy labeling adds to the difficulty by obscuring important cytologic details This brings a particularly important error into DNA labeling, since the label will be taken up by *some* cells of every series (e g , erythroid, myeloid, lymphoid)

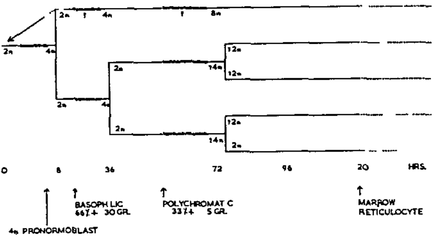

FIG 4—Relation of cell maturation to DNA synthesis and division in the erythroid cells of human bone marrow A pronormoblast with duly diploid amount of DNA 2n) is not easily separated on morphologic grounds from an early basophilic normoblast However *after it has completed the period of DNA synthesis and has reached the 4n complement of DNA* it is easily identifiable After division (cell cycle ~ 18 hours) two morpho*logically identical daughter cells (2n pronormo early basonormo) are formed One of them* retains the capacity for both division and differentiation and subsequently produces 4 re*ticulocytes* The other retains only the capacity for differentiation (but no division) or division (but no differentiation) In the former case it will give rise to one reticulocyte without intervening division in the latter to a pronormoblast Evidence suggests that the ratio of occurrence of the former to the latter is of the order of 1 10

The differences in the length of cell cycle and rate of DNA synthesis or amount of DNA synthesized as illustrated explain the autoradiographic findings of %+ve and grain count figures in different cells The different life span ratios give a number ratio of pronormoblasts basophilic normoblasts polychromatic normoblasts as 0 5 3 16 Whether the non dividing maturing early cell types and the late polychromatic normoblasts contain diploid tetraploid or octoploid amounts of DNA needs to be verified by direct measurements It should be noted that the above is a statistical model thus not excluding any particular pronormoblast to produce (a) two pronormoblasts (b) two dividing basophils or (c) two nondividing basophils

Unless *accurate* differential counts can be made, and unless the proportion of unclassified cells is very small one cannot perform meaningful %+ve or grain count distribution measurements A great deal of this error would be avoided by using a series specific label that is taken up by 100 per cent of the cells of that series e g Fe^{59} in the early erythroid cells and S^{35} sulphate in the early myeloid cells A particularly good example is the work of Alpen[1] with Fe^{59} which will be discussed in a later paper

Certain disadvantages of the tritium label have been discussed in an earlier paper[6] It will be sufficient to repeat here that the radiation effect from tritium localized in the nucleus may not be negligible in certain experiments In attempting to follow a cell through 3 to 4 divisions the original label which after 4 divisions still gives about 20 grains over a cell after 50 days exposure time may easily deliver radiation to the nucleus at the rate of 1 rad/hour Although 1 rad/hour is already a not negligible dose of radiation, the situation with intra nuclear H^3 is further complicated by the geometry A single average H^3 β par

TABLE 2 —*Indications of the Cell Cycle Length in Different Marrow Cells*

Myelocytes	*Blast cells*
35%+ve	18%+ve
31gr/+ve cell	18gr/+ve cell

Lymphocytes show 2 populations

6% large lymphs	94% small lymphs
30%+ve	0%+ve
30gr/+ve cell	—

Note Similar results are obtained irrespective of the label (formate C^{14} adenine C^{14}, thymidine H^3 or P^{32})

TABLE 3 —*Amino Acid Uptake by Bone Marrow Cells (3 hrs in vitro)*
grains/+ve cell corrected for spec act and exposure

	Glycine	Arginine	Lysine	Tyrosine	Phenylalanine
Normal myelocytes 7 expts	94	71	74	65	55
PA myelocytes 4 expts	118	95	92	95	—
Blast cells 4 expts	56	—	70	49	—
Lymphocytes 5 expts	14	10	10	10	10

Note Normoblasts 20 to 25 per cent higher than myelocytes in normal marrows megaloblasts 30 to 35 per cent higher than myelocytes in PA marrows plasmocytes (normal or myeloma) same as lymphocytes

No difference is found between myelocytes from normal as opposed to chronic myelocytic leukemia or lymphocytes from normal as opposed to chronic lymphocytic leukemia patients

ticle will dissipate most of its energy in a volume of 1 μ diameter, and the dose within this volume would correspond to about 170 rads It may depend on chance whether an essential structure has been damaged or not

CONCLUSION

Although some of the disadvantages of H^3 thymidine as DNA label could be avoided by using the more expensive C^{14} thymidine the evidence presented above indicates that DNA labeling may not be the best method for following population dynamics in differentiating systems such as the bone marrow It is suggested that although autoradiography (i e , work at the cellular level) is the essential tool, the label should be chosen according to the system investigated For the erythroid series Fe^{59}, and for the myeloid cells S^{35} sulphate may be preferential, leaving the lymphoid system as the main field of DNA labeling

In a homogeneous population of cells with geometric growth the proportion of cells at any time synthesizing DNA (assuming that all cells synthesize a similar amount at a similar rate) gives an indication of the cycle length, i e , growth rate of the cells In such cases an instantaneous labeling of DNA (e g, with H^3 thymidine) and the scoring of the percentage of labeled cells (%+ve) gives information that is perhaps more meaningful than the mere morphologic appearance of the cells For example, if the clinical course and responsiveness to different courses of treatment of human acute leukemias is correlated with such %+ve data, the information so obtained has a valuable functional basis and a better validity than comparisons based on uncertain morphologic nomenclature The real value of such investigations is in cases where, instead of assumptions, the rates and amount of synthesis are measured by grain counting and following the rate of increase in time of the %+ve figure

This is the type of growth rate determination where DNA labeling is most useful For the more complex analysis of cell population dynamics in differentiating tissue, its use will be limited by the difficulties presented

Finally, in view of the doubts raised in respect to stability of DNA, it is suggested that particular attention be given to the problem in work with DNA labeling of the *erythroid* series of cells These cells have the unusual ability to lose their nuclei in the course of their maturation, and it is still not settled whether this is achieved by karyolysis or extrusion, or both The question of DNA *stability would be very open, dealing with cells capable of lysing their nuclear material*

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Discussion of Leukocyte Production and Survival Using C^{14} and P^{32}

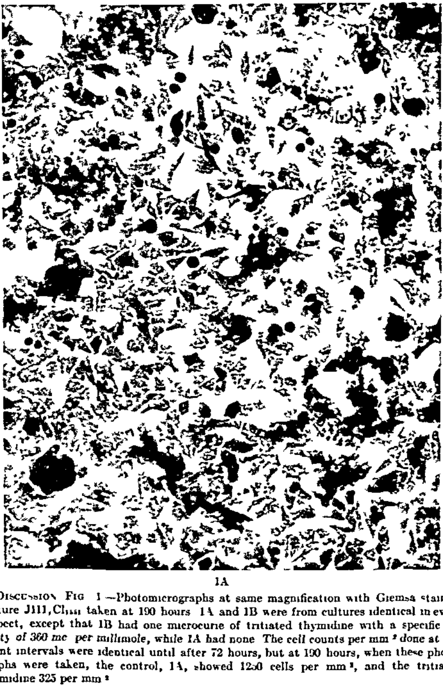
G E CARTWRIGHT It is impossible to speak of the life span of a leukocyte unless one first defines life span, and then indicates which leukocyte he is talking about The life of the granulocyte may be divided into several distinct phases These are (1) mitotic division in the bone marrow, (2) maturation in the bone marrow (3) storage in the bone marrow, (4) circulation in the blood, (5) presumably a period in the tissues, and lastly, as Dr Osgood has pointed out, a period of dying One must define to which of these segments he refers This is extremely difficult for as was previously pointed out, the primitive proliferative pool may be labeled with certain isotopes and one then has to define what he means by "primitive proliferative pool If one takes a very broad look at this we then go back to what Dr Osgood has very aptly called the immortal cell, the one that passes on from individual to individual

Much of the confusion in the leukocyte literature derives from the fact that there is rarely a clear definition of the type of leukocyte studied For example I have not not heard anyone mention the purity of their granulocyte or lymphocyte fractions What has been done in most cases is to

ilton and Cartwright that if there is random loss, this equation does not apply It is also true that if there is an overlap between the period of production and destruction it cannot be used It is quite clear that there is such an overlap in the case of white cells

K E FICHTELIUS I think Dr Kline when speaking of lymphocyte life span meant three different things the time spent in the blood the age of the cells in the blood and finally the life span No one has calculated this last It must be the time from the last division until the death of the cell You can get a rough estimate of the average life span of the lymphocytes through calculating the number of lymphocytes in the whole body and the number formed each day I haven t seen such a calculation until now

I should like to make a little propaganda for a method of isolation of lymphocytes by which you can get 100 per cent of them A glass tube is filled with ordinary cotton wool and heparinized blood is poured into it and incubated in a wet chamber at 37 C for one half hour Then the cotton is carefully washed with saline using a water suction pump The lymphocytes are washed tration of P^{32} there is an initial high peak in the blood which falls, in less than an hour, to a low but in our opinion very significant level Most workers, in calculating life span from DNA P^{32} incorporation, ignore either the entire clearance curve or the plateau portion of the curve and assume that labeling is instantaneous We feel that this cannot be done with our present knowledge of DNA P^{32} incorporation Following a single dose of P^{32}, we don't know whether the significant labeling occurs over a very



1A

Discussion Fig 1 —Photomicrographs at same magnification with Giemsa stain of culture J111, Clion taken at 190 hours 1A and 1B were from cultures identical in every respect, except that 1B had one microcurie of tritiated thymidine with a specific activity *of 360 mc per millimole*, while 1A had none The cell counts per mm ^{2} done at frequent intervals were identical until after 72 hours, but at 190 hours, when these photographs were taken, the control, 1A, showed 1250 cells per mm ^{2}, and the tritiated thymidine 325 per mm ^{2}

short period of time or is continuous over a longer period

No one has mentioned the purity of their DNA or RNA separation methods. Most people use the Schmidt-Tannhauser method, which we have found very unreliable With the *original* method, we have noted a great deal of contamination with RNA, about 25 per cent, depending on the material we worked with Davidson et al[4] have reported similar contamination problems

I noticed that one of the workers gave some of the patients as much as 2 5 mc of P^{32} I think even 0 5 mc or less must produce some damage to the cells and may effect life span calculations To assume that this patient is in a steady state after getting 2 5 mc may not be correct

A final point about the meaning of leukocyte life span" curves, using DNA-P^{32} incorporation In studying a number of patients with myeloproliferative syndromes infections, etc we found that the rate of appearance of the cells may vary In some diseases, such as acute leukemia, there will be an extremely rapid rate of appearance of labeled cells in peripheral blood as compared to the normal. However, the rates of disappearance in these various conditions are approximately the same. We feel that the curve of activity obtained from peripheral blood is not necessarily a measure of the length of time that the labeled cells stay in the peripheral blood. It may only be a reflection of the turnover time in the bone marrow. Dr. Craddock has elaborated on this in his paper.

E. P. CRONKITE: In Ottesen's study[5] only 200 μc of P^{32} was given. Dr. Robertson has looked at the dose and this is extremely small. I do not think that one can suggest any radiation effect in his work.

S. PERRY: I wasn't referring to Dr. Ottesen but to Dr. Kline's paper, where at least one of the patients received 2.5 mc.

L. S. KELLY: It seems to me that the specific activity time curves which Dr. Hamilton finds in leukemic lymphocytes could be due to cell division with long intermitotic times rather than to reutilization of DNA.

E. P. CRONKITE: That was a question that I was going to ask Dr. Hamilton. I think the slope of his curve could reflect the generation time of the most primitive cell that feeds into that line.

K. E. FICHTELIUS: Rosegotti[6] labeled the lymphocytes *in vivo* with Fe^{59}, a cytoplasmic label, and he got the same result as Ottesen who labeled DNA. Therefore, if DNA is reutilized, the whole cell must be reutilized.

L. D. HAMILTON: To answer Dr. Perry, a 25 per cent contamination of DNA with RNA is rather high and must be due to some unsatisfactory procedure. Of course, isolation of DNA itself by detergent procedures is a more clean cut technic, providing precautions are taken to remove RNA by treatment with RNase. In our procedure involving hydrolysis of RNA to nucleotides and of the DNA to free bases, we estimate a maximum RNA contamination of 5 per cent.

I have looked into the possibility that the small percentage of granulocytes in patients with chronic lymphatic leukemia might influence the shape of the radioactivity curves. On the assumption that the RNA and DNA radioactivity turnover curves of granulocytes are identical, I have subtracted that proportion of RNA activity that might be associated with granulocytes from the DNA radioactivity curves of lymphocytes, but we still get two major components and an initial rapid decline followed by a slow decline.

I quite agree with Dr. Fichtelius that if there is reutilization one wonders whether the whole lymphocyte is reutilized. I have emphasized this point previously in relation to our RNA and DNA turnover studies[7] and Rosegotti's results with Fe^{59} underscore this point.[8]

Despite Dr. Kelly's exhortations over the past few years, I have been very unwilling to accept her proposal as an alternative interpretation of our data, chiefly because her idea was that a small lymphocyte somehow or other went back into the lymph node and then divided again.

L. S. KELLY: Yes. The major proportion of the cells that you measured could divide again.

L. D. HAMILTON: I don't think there is too much evidence that small lymphocytes divide, even chronic leukemic lymphocytes. I seem to remember that Dr. Osgood has only seen one small leukemic lymphocyte in division.

It is difficult to answer the question raised by Dr. Cronkite, namely, that the slow slope of the lymphocyte curve could reflect the generation time of the most primitive cell that feeds into that line. Our failure to find any alteration in the radioactivity curves when we perturbed the total lymphocyte mass with steroids or nitrogen mustard a long time after giving the initial label is not inconsistent with this possibility, although our failure to find greater activity in the lymph nodes than in the blood a long time after giving the label would argue against the presence in lymph nodes of a specific primitive cell that feeds into the line. We don't know. We are faced with the problem of a relatively rapid appearance of label in the DNA of lymphocytes and then a very long retention of label. Obviously this must reflect two quite distinct processes.

E. E. OSGOOD: There is a great advantage in maintaining a uniform isotope concentration. In the type of study that Dr. Hamilton showed, you can maintain a constant plasma level of P^{32} and determine over long periods of time the lymphocytic DNA decay rate Deserving of particular emphasis is the study of long term cultures with tritiated thymidine [8, 9] The cells settle on the slide, can be viewed *in situ* and you can count from well distributed areas the absolute number per mm^3 so that your values are in absolute terms and your cells are seen exactly where they are growing The thymidine is not broken down to tritiated derivatives by these human hemic cells as it is in the body by liver cells so it is available for incorporation throughout the experiment

Discussion figure 1 illustrates the fact that there can be such a thing as too much thymidine The counts for controls and cultures exposed to thymidine were identical up to 72 hours, but at 190 hours you can see at a glance extensive radiation effects

The lymphocytes grow in chains in these cultures In chains of lymphocytes cultured from the blood of a patient with chronic lymphocytic leukemia, the end cell has the least label, the next cell more and the first cell the most [8] The last mentioned is the alpha cell, which can divide again This cell divided, produced the terminal cell of the chain, divided again and produced the next cell, etc You wouldn t get such a chain from an alpha 2-alpha division but rather a branching arrangement

K E FICHTELIUS I would like to say to Dr Gowans that there are other problems in the world than the lymphocyte circulation When I started these transfusion experiments I wanted to find out whether there was a difference between thymus lymphocytes and lymph node lymphocytes a very old problem, as you know I have developed a hypothesis that there is an integration between thymus and spleen in antibody formation Transfused P^{32} labeled thymic lymphocytes are recovered perifollicularly in the spleen at the same site as plasma cells are found during antibody formation [10]

E P CRONKITE In considering reutilization, and in fact all of the isotopic studies one should bear in mind that there is some sort of a primitive progenitor pool, either a common pool or a separate one for each cell line Once this is labeled, it is labeled for the rest of the life of the individual The DNA label cannot be lost It can only be diluted by successive divisions Any interpretation of the tail of a curve such as presented by Dr Hamilton must take into account the continued feeding of labeled cells into this pool from the progenitor cells The slope of the curve would reflect in part the generation time of that cell It does not preclude reutilization There may be other factors but this certainly is one

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Cell Turnover in Blood and Blood-Forming Tissues Studied with Tritiated Thymidine

By V P Bond, T M Fliedner, E P Cronkite, J R Rubini and J S Robertson

TRITIATED THYMIDINE (H^3Th) has been used extensively in the study of proliferating cell systems, particularly the hematopoietic system, and pertinent literature has been cited previously [4, 5, 10] The material is incorporated selectively into DNA, and loss of label apparently occurs only through cell death or by dilution through successive cell divisions In patients with apparently normal hematopoiesis, labeling occurs within minutes in the marrow [4, 5] Within days the labeled granulocytes and mononuclear cells (predominantly large) appear in the peripheral blood In vitro studies on blood and bone marrow of normal individuals and of individuals with hematopoietic dysfunctions have been *reported* [2, 3] *The present communication is concerned with in vivo studies on an* individual with apparently normal hematopoiesis, and deals with the quantitative kinetics of the erythrocytic precursor series, studies with H^3Th labeling and the appearance and disappearance of labeled segmented neutrophils in the peripheral blood

Materials and Methods

H^3Th with a specific activity of 1 2 c /mM was used, 1 9 mc of H^3Th was injected intravenously for the bone marrow studies and 0 9 mc for the studies on peripheral blood There was a multicomponent plasma clearance with virtually complete clearance in one hour [5] Autoradiograms were exposed for a uniform period using British stripping film and counterstaining with Wright's or Giemsa Red *cell precursors were divided into five classes the two earliest identifiable stages* were designated B_1 and B_2 the more mature elements were termed basophilic, polychromatic, and orthochromatic normoblasts Criteria were morphologic except in doubtful instances where the measurement of nuclear diameter also was *employed*

Previous investigators[11, 13, 16, 18, 21] have divided the red cell precursor series into five groups the average volume of these cells decreasing by a factor of *approximately 2 with increasing maturity The nuclear diameters as seen in the* stained smear are approximately 13, 10 5 8 25, 6 5, and 5 25 microns respectively

Results

Ratio of erythroid cell precursors Of a total of 2456 nucleated red cell precursors counted by a single individual (pooled data from several samples of bone marrow), 67 were B_1 cells, 146 were B_2 cells, 413 were basophilic normoblasts 549 were polychromatic normoblasts, and 1281 were orthochromatic normoblasts. These are approximately *in the ratio of 1 2 4 8 16, obviously many more* cells and several individuals would have to be counted to establish these ratios with certainty

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From the Brookhaven National Laboratory, Upton, L I N Y

TABLE 1.—*Labeled Erythrocytic Precursor Cells as a Function of Time after H³Th Injection*

Time	B_1 Cells			B_2 Cells			Basophilic Normoblast (B_3)			Polychromatic Normoblast (B_4)			Orthochromatic Normoblast (M_1)		
	No. Labeled	Total Counted	%	No. Labeled	Total Counted	%	No. Labeled	Total Counted	%	No. Labeled	Total Counted	%	No. Labeled	Total Counted	%
½ hr	5	6	83	9	14	64	21	37	57	21	50	42	0	131	0
1 hr	3	3	100	5	7	71	25	29	86	10	25	40	0	66	0
1½ hrs	2	2	100	8	10	80	17	23	74	22	39	56	0	68	0
2 hrs	2	2	100	4	5	80	22	26	85	13	26	50	0	53	0
3 hrs	2	2	100	5	7	71	17	18	95	10	33	30	0	67	0
4 hrs	2	6	33	5	9	56	18	33	55	40	64	63	9	160	6
6 hrs	2	2	100	6	7	86	17	25	68	12	25	48	7	93	8
8 hrs	1	1	100	7	8	88	28	31	90	21	33	64	9	58	16
10 hrs	1	3	33	1	4	25	14	19	74	21	29	72	22	78	28
12 hrs	8	19	42	25	38	66	46	77	60	66	90	73	69	184	37
14 hrs	3	7	43	7	15	47	21	48	44	28	61	46	31	125	25
1 day	1	4	25	3	7	43	9	19	47	12	25	48	37	75	49
2 days	1	5	20	1	5	20	14	24	58	13	32	41	42	100	42
3 days	2	3	67	3	14	21	15	30	50	10	26	38	35	82	43
5 days	0	7	0	2	9	22	1	17	6	0	40	0	0	49	0

Labeled erythroid cells. The number of each cell type counted and the number labeled are shown in table 1. The per cent of labeled cells is shown graphically as a function of time in figure 1 for the basophilic, polychromatic, and orthochromatic normoblasts (B_1 and B_2 cells were not plotted because of the small numbers of cells involved). The percentage of labeled cells increased from time

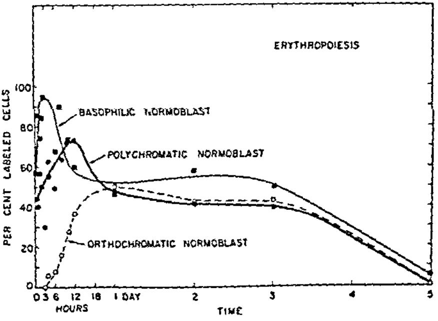

FIG. 1.—Per cent labeled cells in the basophilic, polychromatic, and orthochromatic normoblast groups as a function of time after H³Th injection.

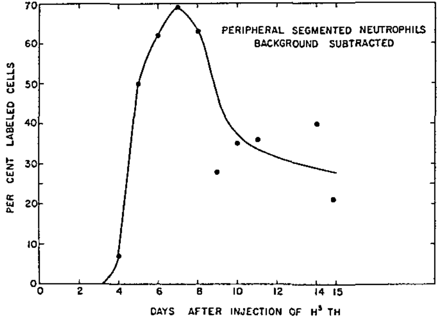

Fig. 2.—Per cent labeled segmented neutrophils in the peripheral blood as a function of time after H^3Th injection. All cells with any grains were counted as labeled, and the measured background was subtracted.

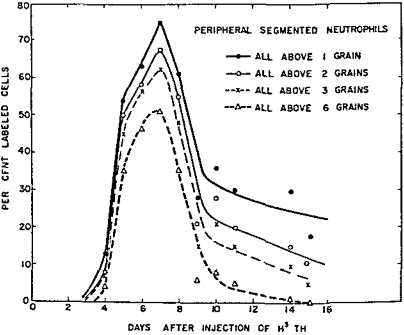

Fig. 3.—Per cent labeled segmented neutrophils in the peripheral blood as a function of time after H^3Th injection. The different curves were determined by considering as labeled only those cells having a number of grains above the arbitrary values stated for each curve in the figure.

0 to 1½ hours in all DNA synthesizing cells The percentage began to fall at 10 to 12 hours, and gradually approached zero at day 5 Labeled orthochromatic cells began to appear at 3 hours, and gradually rose to a plateau at about 12 hours.

Grain counts in all of the erythroid cell series except the orthochromatic normoblast reached a maximum at 1½ hours, before dilution by division had occurred At this time there was a distinct difference in the number of grains over the different cell types, approximately 75 to 100 over the B_1 cell, 40 to 60 over the B_2 cell, 15 to 40 over the basophilic normoblast, and 8 to 20 over the polychromatic normoblast (means of approximately 80, 50, 25, and 12, respectively) At this time there was relatively little spread in grain count. By 6 8, or 10 hours, grain counts were lower, and most data were consistent with two populations of labeled cells, one with a smaller grain count than the other By 24 hours the grain count of all cell types except the orthochromatic normoblast had decreased, and there was a continued reduction in intensity of label with time At 5 days, when there were few labeled cells, the grain counts were uniformly very small

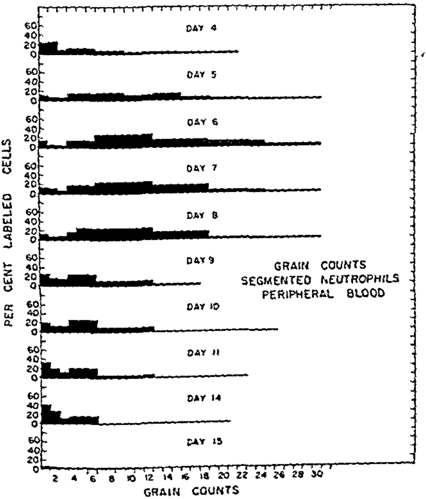

Fig 4 —Graphic presentation of grain counts of mature neutrophils as a function of time after H^{3}Th injection.

TABLE 2.—*Grain Count of Segmented Neutrophil Granulocytes*

Day	Mean	Standard Deviation
4	9.6	3.1
5	12.5	3.5
6	12.6	3.5
7	11.7	3.4
8	11.3	3.4
9	7.0	2.7
10	6.7	2.6
11	6.3	2.5
14	5.4	2.3
15	5.2	2.3

The maximum and mean grain counts of the first generation of orthochromatic normoblasts were equal approximately to those of the initially labeled polychromatic normoblasts.

Peripheral segmented neutrophils. The data shown in figures 2 and 3 represent approximately 400 cells counted per point. Some labeled cells were seen as early as the third day, however, appreciable numbers were not seen until day 4. A steep rise occurred between days 4 and 5. A marked decrease in the percentage of labeled cells occurred between the eighth and ninth days, followed by a slower rate of decline. A comparison of figures 2 and 3 indicates that the shape of the curve is not sensitive to the manner of defining a labeled cell. The curve for the percentage of labeled band neutrophils paralleled exactly that of the segmented forms,[5] but occurred approximately one day earlier.

The distribution of grain counts over the segmented neutrophils is shown graphically in figure 4, and the mean grain counts in table 2. It is clear that different populations of labeled cells were present at different time intervals. The possible significance of this will be considered in the discussion below.

DISCUSSION

Before discussing the results, an oversimplified model of cell proliferation will be presented to indicate what might occur and would be expected as a function of time after H^3Th administration, if the availability time is short (fig. 5). There are five types of dividing precursor cells, which should label within minutes of H^3Th injection, and nondividing cells that do not label initially (maturation only). The latter cells would not be expected to be labeled until division of precursor cells. Within minutes or hours of H^3Th injection, before division of labeled cells has occurred, the percentage of labeling of each type of precursor cell should equal approximately the DNA synthesis time/generation time for that cell type. In the model (fig. 5), the hatched box represents DNA synthesis time, the entire box represents generation time. The average grain counts are represented in the upper left corner of the hatched box, the relative number of cells in each compartment are found in the lower right (the relative numbers of cells indicated would be correct only if all divisions beyond the primitive progenitor (ppp) cell are doubling divisions, and if the generation times in all compartments are equal).

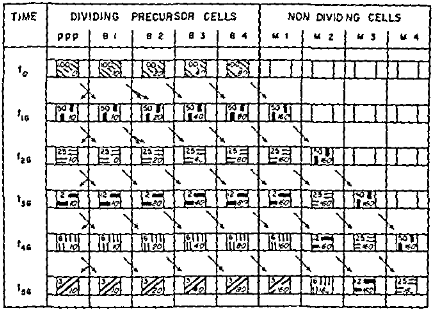

FIG. 5.—Simple model of hematopoietic cell proliferation indicating the course of events at succeeding generation times following administration of H^3Th (see text). Numbers to upper left of boxes indicate expected grain counts if initial average grain count is 100; number to lower right indicates relative number of cells in each compartment.

It is seen that with increasing time (shown in units of generation time in the model), labeled cells will divide and appear in general as two labeled cells in the next more mature compartment. The ppp cell on division, however, will retain one labeled cell as a ppp cell, and only one will mature to form a B_1 cell. With successive divisions the grain counts on the average will continue to halve.

Most important to visualize from the model is that once labeled, all of the precursor cells and their nucleated progeny in theory are labeled indefinitely; thus in theory the labeled cells cannot be "lost." Unless one can conceive of a primitive precursor cell that synthesizes DNA and divides, but does not incorporate H^3Th during DNA synthesis, then the entire generative compartment and all maturation compartments with nucleated cells derived from division of labeled precursors *must continue to be labeled indefinitely.* A compartment can cease to be termed labeled only if (1) on division the grain counts fall below the arbitrary limit at which cells are termed labeled, or (2) there is a selective loss of labeled cells, i.e., a selective effect of the H^3Th or its radiation on the labeled cells. The percentage of labeled cells will change only if one of the above mechanisms is effective, or if the initial percentage of labeled cells of an earlier compartment were different from that of the compartment under consideration.

As expected from the above considerations, all erythroid precursors, except the orthochromatic normoblasts, incorporated H^3Th within minutes of injection of the material, indicating that these cells actively synthesize DNA and presumably can and do divide. About 50 per cent of the polychromatic normoblasts, 75 per cent of the basophilic normoblasts and the B_2 cells, and perhaps even a higher percentage of the B_1 cells labeled initially. It is obvious, as stated above, that the percentage of labeled cells is inextricably bound to the grain count and to a definition of what is termed a labeled cell The lower the average grain count over cells, the fewer cells that will be scored as labeled Some arbitrary number of grains must be selected, depending on background and other factors, below which a cell is considered not to be labeled An examination of the grain count data indicated that this consideration does not materially affect the results, even though the more mature forms have a lower average grain count The only apparent explanation is that the earlier forms have a long DNA synthesis time with respect to generation time

The grain counts observed before cell division had occurred over the several erythroid precursor cells were not equal, and the average grain counts were roughly in the proportion of 80 50 25 12, as one goes from the B_1 cell to the polychromatic normoblast This must be due either to a slower over all rate of incorporation of H^3Th in the more mature forms, or to equal rates of incorporation and differing grain counts resulting from geometrical considerations It has been reported that more mature bone marrow precursor cells have fewer chromosomes than do less mature forms, however, this relatively small difference probably would not account for the different grain counts The nuclear diameters of the precursor cells as seen in the stained slides decrease as one goes from the earliest to the most mature precursor forms Consideration of nuclear size has led several authors to conclude that the nuclear volume decreases by successive factors of 2 as the cell matures, assuming the volumes vary between the square and cube of the diameter observed in the stained smear, the volumes would approximately halve It is difficult to estimate the degree of effect that this changing geometry might have, and one cannot say immediately if, for the same amount of H^3Th contained per cell the smaller nucleus should yield a higher or equal grain count *

A reasonable explanation for the decreasing grain count with increasing maturity is as follows If we consider cells of different diameter, and, thus, surface area and volume suspended in a solution of H^3Th, and assume either an active or passive transfer of H^3Th from the solution to the nucleus, then it is apparent that the amount of H^3Th available to the nucleus for DNA synthesis should vary with the surface area, or perhaps with the volume of the cell In any event, the smaller cell should have considerably less H^3Th, and therefore a smaller grain count In principle one should be able to check these considerations by comparing the grain count of a cell with that of *its progeny* one generation time later Where this is possible (B_1 vs B_2 cells, B_2 cells vs basophilic normoblast, basophilic vs polychromatic normoblast), the numbers of cells are too small for good statistics However, the results are consistent with a halving of grain counts, thus indicating different H^3Th content among the initially labeling cells, and minimizing a geometric factor in the autoradiogram as being responsible for the difference in initial grain counts The only point at which the data obviously deviate is in the comparison of grain counts over the initially labeled polychromatic normoblasts and the first generation of labeled orthochromatic normoblasts. Here the maximum and average grain counts appear to be identical, and the explanation is not apparent. The importance of these considerations in any treatment of grain count data is obvious.

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* In tissue culture, with much larger cells, the smaller cell yields the higher grain count * However, these results cannot be applied directly to the present situation

Information on the generation time of some of the red cell precursors can be deduced from the percentage of labeled cells as a function of time (fig. 1). It is seen that the orthochromatic normoblasts begin to label at about three hours, which would represent the minimum postsynthesis rest period and the mitotic time of the polychromatic normoblast. The percentage of labeled cells continues to increase until a plateau is reached, equal roughly to the initial percentage of labeled polychromatic normoblasts. The 9 hour period of increase approximates the minimum DNA synthesis time of the polychromatic normoblast. No information can be deduced on the postmitotic rest period from present data, however an assumption of 3 to 6 hours would give a minimum generation time for the *polychromatic* normoblast of the order of 15 to 18 hours. This number is consistent with that derived for red cell precursors from the marrow mitotic index.[6]

A more exact estimate of turnover time for the *orthochromatic* normoblast can be deduced from compartment theory. From figure 1, it is possible to obtain the slope of the curve between 3 and 12 hours, which indicates the rate of increase in the relative number of labeled orthochromatic normoblasts in the compartment. This value is 5.3 per cent/hour. Assuming a steady state and what amounts to a "pipeline," then all cells entering the compartment during this interval are labeled and represent the rate of flow of all cells into the compartment. The turnover time of the orthochromatic normoblast then is equal to 100/5.3 = 19 hours. If all cells entering the orthochromatic pool are not labeled at the time the slope is greatest, unlabeled cells are also entering. Then the rate of increase derived from the labeled cells represents a *minimum* rate of cell entry into the pool, and thus the turnover time derived represents an *upper limit* for this value.

No information can be derived directly from the data on the generation time of precursor cells earlier than the polychromatic normoblast. Considerable additional information could be deduced from kinetic theory if the relative numbers of cells in each compartment and the precise shape of the curves of percentage of labeled cells in all compartments vs. time were known with certainty. However, considerably more data than presently are available are required to obtain meaningful results from this type of analyses.

An important consideration involves the apparent rate of rise in the percentage of labeled cells in the several compartments over the first 10 hours, and the final percentage achieved. If anything approaching a "pipeline" is maintained and the generation times are reasonably similar, it would be expected that in one generation time each compartment would have the same percentage of labeled cells as seen in the previous compartment within the first hours of H^3Th administration. Since the earlier compartments show a higher percentage of labeled cells initially, the rise should be sustained for a period of several generations until loss of label by dilution, or unless an unidentified early precursor cell has a relatively low percentage of labeled cells. It is questionable from the data, however, that any erythropoietic precursor, with the exception of the orthochromatic normoblast, reached in one generation time the percentage positivity of its precursor. The rises seen are not sustained beyond one generation time at most. Apparent loss of label by dilution through division cannot be invoked to explain this early loss in percentage of labeled cells A possible effect of the H^{3}Th on these early labeled cells must be considered although other explanations for the findings are possible

In all initially labeling erythroid precursor cells there appeared to be an increase in the number of labeled cells from 30 minutes to 1 or 1½ hours If true this would indicate either that the thymidine is available longer than the few minutes previously proposed (slow marrow circulation?) that reutilization of thymidine occurs or that some storage of H^{3}Th or of intermediate synthesis products occurs before DNA synthesis (stored material would be washed out during processing of the slides)

Independently of the manner in which the percentage of labeled segmented granulocytes in the peripheral blood are plotted (figs 2 and 3) the basic shape of the curve remains the same The upward slope of the curve is equivalent to a 43 per cent rise per day Thus at least this amount of cells must be entering the peripheral blood per day and in the steady state must be leaving the blood each day If all cells entering during this time are labeled which would be true if a pipeline approximated the situation then the average life time of a mature granulocyte (turnover time average sojourn time) in the peripheral blood would be 100/43 = 2 3 days in the steady state If unlabeled cells are also entering the blood during the steep rise in the curve the value of 2 3 days derived would be greater than the true value for the turnover time Thus the value of 2 3 represents an *upper limit* for the average turnover time of the mature neutrophils in the blood The actual value could be much less than this value the method of analysis indicates that it cannot be more Note that this figure indicates the average time spent in the peripheral blood (turnover time average sojourn time) It is not necessarily equal to a mean life span for the individual leukocyte If there is an extravascular pool of leukocytes as has been postulated and granulocytes do not return to the blood[6] then of course segmented neutrophils could move into this pool and the mean life span of the individual leukocyte would be longer than indicated by the figure given

While the upward slope of the curve for labeled segmented neutrophils in the peripheral blood is amenable to interpretation the downward slope(s) offers considerable difficulty This will be discussed after further consideration of the simplified model (fig 5) and of grain counts which bear heavily on the interpretation

From the model it is seen that labeled mature cells should appear in the peripheral blood after passing through metamyelocyte and band forms They would remain labeled for the duration of their stay in the peripheral blood The *percentage* of labeled cells in the blood should build up to roughly that of precursor cells and should theoretically remain at the level of the earliest precursor Label should be no longer visible only when diluted sufficiently by precursor division With regard to grain count if the percentage labeling of all precursor cells is equal then one might expect to see waves of labeled neutrophils each with apparently ½ the grain count of the previous lot With the determined peripheral blood life time of approximately two days or less equivalent roughly to the generation time of the dividing precursor cells one would expect to see overwhelmingly one grain count population during a generation time of the pre cursor cells perturbed to a relatively minor degree by preceding or succeeding waves. The dividing myeloid precursor cells, however, do not have the same average grain count. The myelocyte has half or less of the grain count of the promyelocyte (adequate data are not available on the myeloblast). Thus, a halving of the grain count after one generation would not be expected. On examining the data (fig. 4), it is seen that the grain counts on days 5 and 6, when the percentage of labeled cells is building up to a maximum, are consistent with a single grain count population. The grain count populations on days 7 and 8 appear to be different from those on days 5 and 6, as are the populations on days 9 and 10. The increased average grain count on days 7 and 8 over that on days 5 and 6 can be explained if the latest dividing precursor cell has half, and probably less than half, the grain counts of the next earliest cell (which on two divisions has the same or greater counts than the last dividing precursor after one division). No information on the expected grain count of later waves can be deduced, since more than the last two precursor cells have not been studied.

It is of considerable importance to consider grain counts in conjunction with the percentage label curve (figs. 2 and 3) at the time the curve is falling off rapidly (days 8, 9, and 10). A reduction in the *percentage* of labeled cells can result only from dilution through precursor division, from an unknown precursor with a lower percentage of labeled cells, or from selective loss of labeled cells. With regard to the first possibility, it is seen that on day 8, only a very small percentage of the total cells have grain counts of the order of 1, 2, or 3. Thus, halving of the grain counts from a precursor with the same or greater grain count than the early precursor for the neutrophils of day 9 would not account for an appreciable loss of cells—certainly not the 35 per cent observed. A precursor cell with less percentage label is possible; data on this point are not available. Selective removal of cells, perhaps from a H^3Th radiation effect, is discussed below. The "tail" of the curve can be accounted for adequately by "loss" of labeled cells by successive division of precursor cells and grain count dilution.

No information has been deduced from the present data regarding the presence or absence of an extravascular neutrophil pool; however, data can be derived regarding the possibility of *recirculation* of segmented neutrophils. The grain counts of the segmented neutrophils as a function of time are shown in figure 4. Until day 8, segmented forms with high grain counts are seen; on day 9 and later, the high grain count forms are absent. The data indicate that, if there is normally an extravascular life span and recirculation, the extravascular life span of the segmented neutrophil must be less than 24 hours.

Additional deductions can be made from the grain count data. The fact that the heavily labeled cells seen on day 8 are not found on day 9, indicates that the value for turnover time of the granulocyte is less than the upper limit of 2.3 days deduced from the ascending limb of the curve of percentage of labeled cells. The value is 24 hours or less. More frequent sampling should allow one to obtain a better estimate of turnover time from the grain count data. In theory, the slope of the gradual fall-off in mean grain count after day 8 should yield information on the generation time of the earliest myeloid percursor cell. In practice, however, one cannot follow the mean grain count, since one, of course, cannot score below 1 grain count. The time spent in the peripheral blood calculated for the loss in percentage of labeled cells. A possible effect of the H^3Th on these early labeled cells must be considered, although other explanations for the findings are possible.

In all initially labeling erythroid precursor cells, there appeared to be an increase in the number of labeled cells from 30 minutes to 1 or 1½ hours. If true, this would indicate either that the thymidine is available longer than the few minutes previously proposed (slow marrow circulation?), that reutilization of thymidine occurs, or that some storage of H^3Th or of intermediate synthesis products occurs before DNA synthesis (stored material would be washed out during processing of the slides).

Independently of the manner in which the percentage of labeled segmented granulocytes in the peripheral blood are plotted (figs. 2 and 3), the basic shape of the curve remains the same. The upward slope of the curve is equivalent to a 43 per cent rise per day. Thus, at least this amount of cells must be entering the peripheral blood per day, and in the steady state must be leaving the blood each day. If all cells entering during this time are labeled, which would be true if a "pipeline" approximated the situation, then the average life time of a mature granulocyte (turnover time, average sojourn time) in the peripheral blood would be 100/43 = 2.3 days in the steady state. If unlabeled cells are also entering the blood during the steep rise in the curve, the value of 2.3 days derived would be greater than the true value for the turnover time. Thus the value of 2.3 represents an *upper limit* for the average turnover time of the mature neutrophils in the blood. The actual value could be much less than this value; the method of analysis indicates that it cannot be more. Note that this figure indicates the average time spent in the peripheral blood (turnover time, average sojourn time). It is not necessarily equal to a mean life span for the individual leukocyte. If there is an extravascular pool of leukocytes, as has been postulated, and granulocytes do not return to the blood,[6] then, of course, segmented neutrophils could move into this pool, and the mean life span of the individual leukocyte would be longer than indicated by the figure given.

While the upward slope of the curve for labeled segmented neutrophils in the peripheral blood is amenable to interpretation, the downward slope(s) offers considerable difficulty. This will be discussed after further consideration of the simplified model (fig. 5), and of grain counts which bear heavily on the interpretation.

From the model it is seen that labeled mature cells should appear in the peripheral blood after passing through metamyelocyte and band forms. They would remain labeled for the duration of their stay in the peripheral blood. The *percentage* of labeled cells in the blood should build up to roughly that of precursor cells, and should theoretically remain at the level of the earliest precursor. Label should be no longer visible only when diluted sufficiently by precursor division. With regard to grain count, if the percentage labeling of all precursor cells is equal, then one might expect to see waves of labeled neutrophils, each with apparently ½ the grain count of the previous lot. With the determined peripheral blood life time of approximately two days or less, equivalent roughly to the generation time of the dividing precursor cells, one would expect to see overwhelmingly one grain count population during a generation time of the pre cursor cells, perturbed to a relatively minor degree by preceding or succeeding waves The dividing myeloid precursor cells, however, do not have the same average grain count The myelocyte has half or less of the grain count of the promyelocyte (adequate data are not available on the myeloblast) Thus, a halving of the grain count after one generation would not be expected On examining the data (fig 4), it is seen that the grain counts on days 5 and 6, when the percentage of labeled cells is building up to a maximum, are consistent with a single grain count population The grain count populations on days 7 and 8 appear to be different from those on days 5 and 6, as are the populations on days 9 and 10 The increased average grain count on days 7 and 8 over that on days 5 and 6 can be explained if the latest dividing precursor cell has half, and probably less than half, the grain counts of the next earliest cell (which on two divisions has the same or greater counts than the last dividing precursor after one division) No information on the expected grain count of later waves can be deduced, since more than the last two precursor cells have not been studied

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Additional deductions can be made from the grain count data The fact that the heavily labeled cells seen on day 8 are not found on day 9, indicates that the value for turnover time of the granulocyte is less than the upper limit of 2 3 days deduced from the ascending limb of the curve of percentage of labeled cells The value is 24 hours or less More frequent sampling should allow one to obtain a better estimate of turnover time from the grain count data In theory, the slope of the gradual fall-off in mean grain count after day 8 should yield information on the generation time of the earliest myeloid percursor cell In practice, however, one cannot follow the mean grain count, since one, of course, cannot score below 1 grain count The time spent in the peripheral blood calculated for the band neutrophil was the same as that for the segmented neutrophil, and the additional considerations applied to the segmented neutrophil apply in general to the band neutrophil as well.

Some of the data presented above are consistent with a selective loss of labeled cells from some of the precursor pools. There is ample grounds from iron balance studies[17] and from observed phagocytosis of precursor cells to suspect that such a loss occurs normally.[1] Rigas[14] has incorporated such loss into his model. However, if conditions are physiologic, unlabeled cells as well as labeled cells should be removed. Thus, such a normal removal mechanism would not explain all of the present findings, and one must consider the possibility that radiation from the H^3Th may have perturbed the systems studied. It must be recognized that erythroid precursors are among the most radiosensitive cells. While it is simple to calculate an average dose from the injected H^3Th (the dose is negligibly small) such a calculated average value is meaningless, because the material is taken up selectively in the nucleus and tritium has a short range. One must therefore rely on actual, observed effects. An estimate of the isotope dose level at which effects might be expected can be derived as follows: from tissue culture studies the minimum dose at which an effect can be detected is 0.02 μc/gm of H^3Th of specific activity of 1 c/mM, in the medium for 24 hours.[8] The criterion of effect was delayed or inhibited cell division. The material was available throughout the DNA synthesis period, or for about 4 hours. It is difficult to estimate an average or "effective" availability time in the in vivo studies in man, however 10 minutes or ⅙ hour is reasonable. Thus, some effects might be expected with high specific activity material at approximately 24 × 0.02, or approximately 0.5 μc/Gm. It is at this dose level that it is possible to just detect an effect of thymidine on the extremely sensitive spermatogonia of mice.[19] The dose used in the patient studied was approximately ⅓ μc/Gm of lower specific activity, equivalent in effect, presumably, to about ⅙ μc/Gm of high specific activity material. Therefore, no detectable effects would be expected. In these studies there has been no evidence of abnormal mitosis, and no evidence of a change in the total leukocyte blood count following H^3Th administration. The question of possible radiation effects is of considerable importance to all studies in which DNA labeling is employed, and additional investigations to settle the point are needed urgently.

In the present studies the assumption of a pipeline condition has been made, at least to the extent that all cells in one generative or maturative compartment are in the next compartment, within one generation time. Evidence for this is that all cells initially labeled in the polychromatic pool can be accounted for as double their number in the orthochromatic compartment one generation later. Some evidence for this is also seen from the segmented neutrophils in the peripheral blood, where the grain count population appears to change at one- to two-day intervals or less.

SUMMARY

Data are presented on the time course of labeling of erythropoietic precursor cells and of peripheral segmented neutrophils following the intravenous injection of tritiated thymidine into a patient with apparently normal hematopoiesis.

Four distinguishable erythroid precursors, including the polychromatic normoblast, label directly, the orthochromatic normoblast labels only by division of a precursor cell By five days, essentially all label has disappeared from the precursors, presumably by apparent loss of label through cell division A consideration of the rate of rise in the per cent of labeled orthochromatic normoblasts indicates that the turnover time for this cell type must be roughly 19 hours *or less* The time curve for the percentage of labeled segmented neutrophils in the peripheral blood is analyzed From the rate of appearance of the cells the average turnover time of the mature neutrophil in the blood stream must be 2 3 days *or less* A similar value holds for the band neutrophils Analysis of grain count data indicate that the average turnover time of the mature neutrophils in the peripheral blood probably is 24 hours *or less* Evidence is presented that suggests, but does not prove, that radiation from the intranuclear tritium may have perturbed the earlier precursor cells in the systems studied, further investigations to settle this question are needed

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Kinetics of Neutrophil Balance

By HARVEY M. PATT AND MARY A. MALONEY

MANY CELL SYSTEMS undergo continual renewal with a balance between production and decay.[4] Bone marrow constitutes such a system, and statistical approximations of the over all balance have been made, utilizing general principles of steady state cell populations.[7, 8] The relationships applying to dynamic cell systems have been discussed recently in some detail by Quastler and Sherman.[10]

In this communication, data are presented pertaining to the kinetics of neutrophil balance as revealed in studies of eight normal beagle dogs. The investigation is based on radioautographic analysis of marrow and blood after administration of tritium labeled thymidine, which has the advantages of brief availability, DNA specificity, and high resolution.[3] Experimental procedures were described previously.[5] The present results confirm and amplify our earlier observations[6, 9] with regard to neutrophil production and maturation in marrow, and disappearance from blood.

The tritium label appears first in cells capable of mitosis. The percentage of myeloblasts and promyelocytes labeled initially (30 minutes) appears to be about three times that of myelocytes. This can be attributed, in part, to the fact that all myelocytes do not participate in cell division, as will be shown later, perhaps 40 per cent of cells classified as myelocytes are in a transitional stage between the last mitosis and emergence as a metamyelocyte. The relative number of cells in such a transitional stage will, of course, depend upon the cytologic criteria for differentiation of myelocyte and metamyelocyte.

Details of the generation cycle may be obtained from the temporal distribution of labeled mitotic figures following injection of tritiated thymidine. A correlation of this sort is being made, and the approximate sequence of events may be discerned from the initial analyses in two dogs. It has been found that mitotic figures for myeloblasts, promyelocytes, and myelocytes are labeled by 30 minutes after thymidine administration. These are prophases, labeled cells in metaphase, anaphase, and telophase appear by 1.5 hours. The fraction of labeled mitoses reaches an asymptote between 2 and 3 hours and begins to decrease between 5 and 6 hours. This general pattern, depicted in figure 1, was observed in both dogs, a second increment of labeled mitotic figures was apparent in one of these dogs between 14 and 16 hours after thymidine administration. It will be noted that only about 55 per cent of mitoses were labeled during the first several hours after thymidine injection. We have no explanation for this unexpectedly low value, which requires further study, among the factors that could contribute to it are the variance of the period between DNA synthesis and mitosis, the pos-

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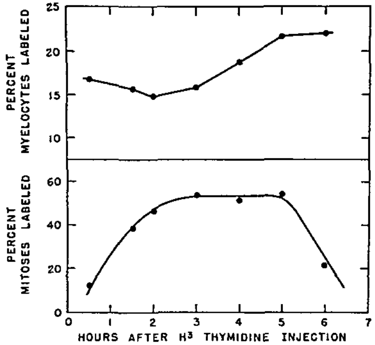

FIG 1 —The initial pattern of labeled myelocytes in a dog Each point in the lower curve is based on at least 50 myelocyte mitoses in the upper curve on a minimum of 1000 myelocytes

sibility of nonuniform availability of precursor, and false negatives (criterion of positivity and smear thickness)

As shown in figure 1, the increase in the fraction of labeled myelocyte mitoses is followed closely by an increase in the per cent of labeled myelocytes Although all cells labeled initially would be expected to undergo mitosis, the increment in labeled myelocytes at 6 hours is only about 40 per cent This can be accounted for, in part, by the fact that all labeled myelocytes have not yet completed mitosis However, loss of minimally labeled cells as a consequence of mitosis is an important contributing factor Some 20 per cent of myelocytes labeled initially have between 4 and 9 grains over the nucleus most of these cells would not be scored as labeled after the first mitosis, since the criterion of positivity has been taken as 4 grains ($p < 01$) The suggestion of an early transient decline in the percentage of labeled myelocytes and the exaggerated lag period would be consistent with less extensive labeling in those cells nearing completion of DNA synthesis, such cells would be expected to enter mitosis first

Although these mitotic data are not adequate for precise characterization of the myelocyte generation cycle certain inferences can be made It would appear that mitosis begins within an hour after completion of DNA synthesis. The combined duration of the postsynthetic period and mitosis is about 2 5 hours Since half of the labeled mitoses are found between 1 and 6 hours in the various proliferating cells, the DNA synthetic period may be approximated from these data as 5 hours Preliminary observations indicate a similar synthetic period for myeloblasts-promyelocytes The fraction of cells labeled initially should be ap

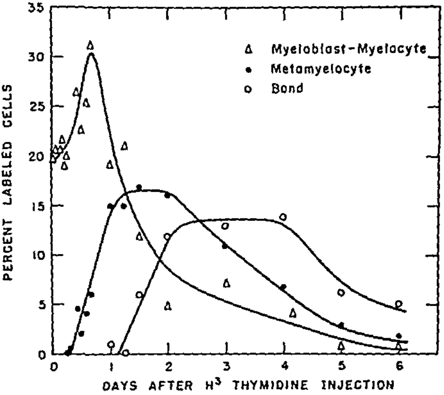

Fig 2—Temporal distribution of labeled myeloid cells in marrow The values are based on differential analysis of a minimum of 1000 cells in each of 4 dogs

proximately equivalent to the fraction of time spent in synthesis, a turnover time of 9 hours for myeloblasts and promyelocytes (considered as a single population) and of 29 hours for myelocytes may be calculated from this relationship Turnover time is equivalent to effective generation time if all cells belong to the progenitor population, this is not the case for myelocytes

The progress of the label from the less to the more differentiated cells is shown in figure 2 Labeled metamyelocytes appear several hours after injection of tritiated thymidine, with an average time of about 16 hours estimated from the ascending curve If we deduct the time from the midpoint of DNA synthesis to completion of mitosis (*ca* 5 hours), then 11 hours will be required on the average for transition of a myelocyte to a metamyelocyte Since these 11 hours are included in the previous estimate of the myelocyte turnover time, the mean generation time for the effective progenitor population of myelocytes will be 18 hours This is in fair agreement with the time of the second increment of labeled mitoses in the one dog noted above

The myelocyte turnover time can be estimated in another way The average time spent in the metamyelocyte class calculated from the data in figure 2, is 20 hours The distribution of myeloblasts and promyelocytes, myelocytes, metamyelocytes, and band cells in marrow aspirates is approximately 1 4 3 4 In the steady state, in which the number of cells in a recognizable stage remains constant, the time spent in a given stage will be determined by the ratio of the number of cells to the number leaving in unit time The flux must be equal for myelocytes, metamyelocytes, band and segmented cells, assuming that few cells die or escape directly into the periphery, somewhat fewer myeloblasts and promyelocytes will enter the myelocyte class in the same time, owing to proliferation within the latter With a metamyelocyte time of 20 hours, the mean myelocyte turnover time will be about 27 hours ($\frac{4}{3} \times 20$,) which is in good agreement with the independent estimate of 29 hours The myelocyte metamyelocyte transition time was previously estimated as 11 hours, hence, the mean myelocyte generation time on this basis would be 16 hours

An appreciable number of labeled segmented neutrophils can be detected in the aspirated marrow within 2 to 3 days after injection of tritiated thymidine This time may be compared with the calculated value of 63 hours from the mid point of DNA synthesis (16 hours to metamyelocyte, 20 hours metamyelocyte 27 hours band cell) Dilution of marrow with blood during aspiration will influence the percentage of segmented cells, the effect on band cells will be negligible Analysis of marrow particle smears reveals that about 15 per cent of the granulocytes are segmented, in contrast to from 20 to 25 per cent in most of the aspirated samples If 15 per cent is taken as a reasonable value, then the segmented to band cell ratio will be around 0 55, and the mean time in marrow for segmented cells will be 15 hours (0 55 × 27)

The various estimates of the chronology of neutrophil development in marrow are summarized in table 1 Despite the provisional nature of the data and the crudity of the analyses, it is of interest to note the close correspondence between the calculated values and the sequence of events in blood The average time for the appearance of labeled neutrophils in circulation was computed as 78 hours (from the midpoint of myelocyte DNA synthesis to release from marrow), the estimated time from myeloblasts and promyelocytes was 99 hours It will be recalled from figure 2 that the peak of all labeled progenitor cells occurred about 16 hours after injection of tritiated thymidine Hence, we would anticipate substantial numbers of labeled neutrophils after the third day, with a profound increase around the fourth day The neutrophil turnover in blood is shown in figure 3 These analyses, based on enumeration of 2000 neutrophils in daily samples from each of 4 dogs, reveal a consistent blood pattern, with labeled cells first appearing 3 days after thymidine injection and rising to a maximum between 4 and 5 days

Estimates of the time spent by neutrophils in blood vary from minutes to days,

TABLE 1 —*Estimates of Chronology of Neutrophil Development in Bone Marrow*

Myeloblast Promyelocyte	Myelocyte		Metamyelocyte	Band	Segmented
	Generation	Transition			
9 hrs	16 hrs 18 hrs	11 hrs	20 hrs	27 hrs	15 hrs

Myelocyte (midpoint DNA) ——Marrow Segmented 78 hrs
Myelocyte ——Marrow Segmented 90 hrs
Myeloblast Promyelocyte ——Marrow Segmented 99 hrs

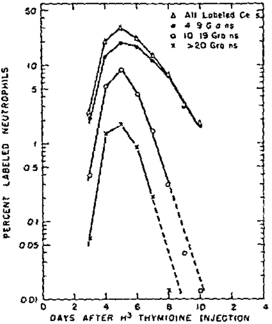

FIG 3.—Labeled neutrophils in peripheral blood The mean values are derived from enumeration of 2000 neutrophils in daily samples from each of 4 dogs

depending upon experimental procedure and analysis This uncertainty raises important questions about the number of vascular and peripheral neutrophils in relation to the number circulating The disappearance of tritium labeled neutrophils is approximately exponential after the sixth day, with a half time of about 30 hours (mean time, 43 hours) When labeled neutrophils are classified by grain count, there are sharp differences in the initial rates of decay, with a half time of 12 hours (mean time, 17 hours) for the more highly labeled cells As shown in figure 3, the latter disappear before cells with minimal labeling (4 to 9 grains over nucleus), which apparently continue to be released in substantial numbers for several days It is generally believed that the label can be decreased only through mitosis The shift in the distribution of labeled cells in the peripheral blood is consistent with the rather progressive decline in mean grain concentration of proliferating and maturing cells in marrow over several days after injection of tritiated thymidine

The level of labeled cells in blood reflects the balance between the number of labeled cells entering and leaving the circulation in unit time In consequence, estimates of the time spent in blood, which are based on over all rates of appearance or disappearance, may be considerably in excess of the true value This is clearly shown by comparison of the rates of disappearance of cells differing in degree of labeling As noted above, the dichotomy between the flux of cells entering and leaving the blood is sharpened by focusing attention on more nearly homogeneous classes of cells as revealed by grain count There is reason to believe that the mean time in blood may be even less than that estimated in this way from daily samples Frequent sampling (*ca* 4 hours) has been made during the apogee of labeled neutrophils in blood Preliminary results in two dogs are indicative of a cyclical pattern of increase and decrease in the percentage of labeled cells during this period, with the peaks separated by 15 to 20 hours and a half time for disappearance of perhaps 6 hours (mean time, 8 hours) These findings, which require further study, suggest that labeled cells may be released more or less synchronously for two to three generation cycles before the aggregate variability of the various phases of neutrophil development becomes significant Whether or not this is indeed the case, it would appear that the mean time spent in blood by neutrophils is a matter of hours rather than days Although it is not yet possible to assign a precise value for the mean time of segmented neutrophils in blood, the present results based on daily sampling reveal a maximum of 17 hours, with the suggestion of a mean time of the order of 8 hours in the two dogs studied at more frequent intervals

Since the mean time for segmented neutrophils in blood seems to be about half of that in marrow, a proportionate number of segmented cells should be represented in the two compartments There is considerable uncertainty about the number of segmented neutrophils in marrow relative to blood If the ratio of segmented neutrophils in marrow and blood is assumed to be between 5 and 10[10] then, on the basis of present estimates of the relative duration in the two compartments, we may calculate that 2 to 4 times as many cells as are detectable in blood by conventional methods must be in a dynamic equilibrium with those that are circulating A large part, if not all, of these neutrophils may exist within the confines of the vascular system, perhaps to a considerable extent as marginal cells[13] If exchange of neutrophils between blood and extramedullary tissue is minimal, a point which apparently receives support from several lines of investigation,[1, 2, 11] then it is not possible to make inferences about the number of tissue neutrophils from the temporal distribution of labeled cells in peripheral blood It should be emphasized that earlier postulates[7, 8] of a very large, exchangeable peripheral pool represented an attempt to resolve the apparent inconsistency between the number of circulating neutrophils and the relatively long time span inferred from DNA P^{32} blood studies[5] That the latter may have been an over estimate has been pointed out[1, 8] and is clearly indicated by the present studies of the fine structure of the blood curve

The exponential decline of blood neutrophils, particularly of the relatively homogeneous classes of labeled cells, is suggestive of a random loss from the circulation Although one is inclined to assume ' a first in—first out ' pattern during the course of neutrophil development in marrow, there is probably an appreciable variability of the source of labeled cells after the first few generation cycles While this variability in marrow could contribute to the exponential disappearance of labeled cells from blood, it would not appear to be a major factor The several questions of neutrophil time span and randomness, which have been alluded to, may be resolved ultimately upon closer inspection of the temporal course of labeled cells in marrow and blood

SUMMARY

Neutrophil development in the normal steady state has been studied radio autographically in eight beagle dogs after administration of tritiated thymidine The several parameters of neutrophil production, maturation, and disappearance are being evaluated in sequential analyses of marrow and blood Provisional esti

mates have been made of the turnover of the various proliferating and differentiating stages in marrow The average time from the midpoint of DNA synthesis in myelocytes to release from marrow was computed as 78 hours, and from myeloblasts and promyelocytes as 99 hours There is a close correspondence between these values and the sequence of events in blood

The blood pattern has been characterized by frequent sampling, with emphasis placed on the disappearance of relatively homogeneous classes of cells as revealed by grain count Although it is not yet possible to assign a precise value for the mean time of segmented neutrophils in blood, the present results reveal a maximum of 17 hours, with the suggestion that the actual mean time may be 8 hours or less The data point to a random disappearance of neutrophils from blood, and indicate that the exchangeable peripheral pool is considerably smaller than previously inferred

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Preliminary Autoradiographic Observations of Cellular Proliferation in Lymphoid Tissues, Using Tritiated Thymidine

By JOHN C. SCHOOLEY, B. J. BRYANT AND LOLA S. KELLY

MUCH HAS BEEN LEARNED about cell proliferation with the use of classical histologic methods as well as with technics involving isotope incorporation into DNA.[11] Autoradiography following the administration of a radioactive DNA precursor, combines some advantages of each approach. In principle autoradiography permits the identification of the proliferating cells, their generation times, and their fate after differentiation. This technic overcomes one of the difficulties inherent in biochemical studies of DNA synthesis, where it is not possible to determine whether all the activity is in a small percentage of the cell population or whether some activity is in each cell.

Autoradiography seemed a particularly useful tool in the study of the leukopoietic system.[10] The diffuse nature of this system and the mixture of proliferating and differentiated cells in some parts of the body but not in others have made biochemically derived turnover times difficult to interpret. It is our task here to describe the results of an autoradiographic study of lymphocytopoiesis in mice and rats. The experiments are still in an exploratory stage, and much revision will undoubtedly be necessary as more data are accumulated. To date much of the information derived merely confirms well-established histologic observations but better quantification and some new facts should eventually emerge. Initially we used adenine-8 C^{14} as the nucleic acid precursor but the availability of tritiated thymidine has made it the tracer of choice and in all our recent experiments we have used this more specific precursor.

The basic premises involved in the use of tritium labeled thymidine and in the interpretation of data obtained with it have been discussed by Hughes et al.[8] and Cronkite et al.[6] (a) thymidine is a specific precursor for DNA which is available for only a short time after injection (b) thymidine is incorporated into DNA only at the time of synthesis, in preparation for cell division and the activity is distributed to the daughter cells at mitosis (c) in a nondividing cell formed from a labeled cell, the activity is stable for the life of the cell. One further assumption which has not yet been adequately studied, is that the dosage of radioactivity used does not influence future biologic potentialities of a labeled cell.

MATERIALS AND METHODS

The *in vivo* studies were performed on young A strain mice with an average weight of 22 Gm. The animals were injected intraperitoneally with 1 μc./Gm. tritiated thymidine (360 mc./mM, Schwartz Laboratories), sacrificed at various

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intervals after injection, and smears made of blood, lymph, bone marrow, and various lymph nodes Lymph was collected from the thoracic duct either at its junction with the jugulosubclavian angle or from the cisterna chyli The results from two different series of mice are reported, and in those cases where samples were prepared at the same time after injection the values were averaged In most cases the values agreed quite well, in a few cases they varied by as much as a factor of three

The in vitro incubations were performed on tissues obtained from Sprague Dawley male rats with an average weight of 224 Gm Suspensions of the thymus and mesenteric lymph node cells were made by gently shaking small pieces of tissue in heparinized serum in the cold, followed by filtration through nylon mesh Lymph was collected from the thoracic duct of several anesthetized donor rats and pooled Blood was collected from the abdominal aorta and incubated as whole blood, or the buffy coat was removed by centrifugation (1000 rpm for 15 minutes) and resuspended in serum The total cell count was adjusted to about 20,000 cells/cu mm, and 15 mg streptomycin sulfate and 1000 units of potassium penicillin G were added to each sample The cell suspensions were placed in sterile 50 ml flasks in a water bath at 36°–37°C and gently shaken for 10 minutes Then tritiated thymidine was added to give a final concentration of 2 μc/ml Samples were withdrawn from each cell suspension and smears prepared at various intervals Throughout the experiment only a low percentage of cells were stainable with eosin

Smears were fixed in absolute methyl alcohol for several hours To prevent loss of lymphocytes, which often occurs with lymph smears, these slides were fixed in 1 per cent gelatin methyl alcohol Sections were fixed in neutral buffered formalin, imbedded in paraffin, and 4 to 5 μ sections were cut

Autoradiographs were made of all smears and sections, using British Kodak Ltd AR 10 stripping film The technic of preparing the autoradiographs was that of Pelc[14] Smears from the in vivo and in vitro experiments were exposed 30 and 14 days, respectively

The autoradiographs were examined, and the percentage of labeling in the different classes of cells determined Cells having less than 3 silver grains above representative background areas were arbitrarily considered to be unlabeled In most experiments separation of lymphocytes into small, medium, and large size categories was done subjectively, but in some experiments, the actual nuclear diameter of each lymphocytic cell was determined, and from these measurements and the differential distribution of cell sizes, a separation of small (5 to 7μ), medium (8 to 9μ) and large lymphocytes ($\geq$ 10μ) was made Because considerable variation may occur in the distribution of different sized cells at the periphery and center of smears, an attempt was made to examine cells in various regions of the slide in a random fashion, and in many cases several slides were examined At least 500 small lymphocytes were examined in the blood smears and 1000 in the thymus and node smears Additional larger cells were often counted to obtain a better sampling of the medium and large size categories

It has been assumed that the number of grains above a cell is proportional to the amount of activity in that cell It is recognized, however, that there are still many unknown variables in autoradiography The sensitivity of different batches of film, the factors that influence latent image formation, and the possible changes produced by staining may alter the number of grains observed over a cell in a nonproportional manner

DNA specific activities were determined at 2 and 5 hours after the intraperitoneal injection of 20μc of $Na_2HP^{32}O_4$ Tissues from 4 to 6 mice were pooled for each sample and the DNA isolated [17] Cell numbers per organ were determined by dividing the DNA phosphorus content by the amount of DNA phosphorus per average cell

In Vivo Labeling of Lymphoid Tissue

The temporal course of thymidine labeling in various lymphoid tissues and blood for the first week after injection is summarized in figures 1 and 2 The data have been plotted to show the difference in the percentage of cells labeled in two size categories and to allow a comparison of different tissues In terms of the total number of labeled cells, it must be remembered that there are 10 to 20 times as many small cells as there are medium sized cells In spite of the considerable scatter among the points, some general trends are evident The larger cell classes initially contained a high percentage of labeled cells, consistent with the known high mitotic index [9, 16] In contrast, only a low percentage of the small lymphocytes contained label, indicating a relatively low turnover for these cells. These observations are in general agreement with work currently in progress in other laboratories [6, 19] and with our adenine-8-C^{14} studies [3] In figure 1, the percentage of total cells labeled is plotted, in figure 2, only those cells with more

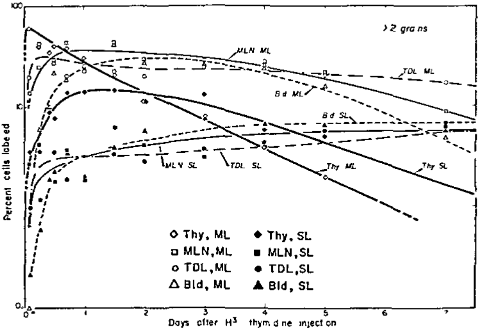

FIG 1 —Labeled lymphocytes in smears of lymphoid tissue and blood Open symbols represent per cent of medium sized lymphocytes (ML) with more than 2 grains Solid symbols represent per cent of small lymphocytes (SL) with more than 2 grains Thy = thymus MLN = mesenteric lymph node, TDL = thoracic duct lymph, Bld = blood

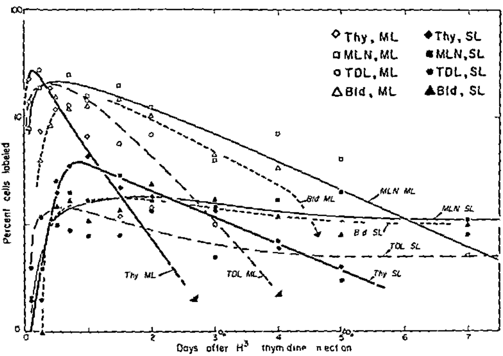

Fig 2.—Heavily labeled lymphocytes in lymphoid tissue and blood. Open symbols represent per cent of medium sized lymphocytes (ML) with more than 10 grains. Solid symbols represent per cent of small lymphocytes (SL) with more than 10 grains. Thy = thymus. MLN = mesenteric lymph nodes. TDL = thoracic duct lymph. Bld = blood.

than 10 grains are included. A comparison of the two figures shows that the percentage of the more highly labeled medium cells decreases more rapidly than does the percentage of all labeled medium sized cells. This is to be expected since the labeled DNA would be diluted with successive divisions.

Thymus and mesenteric nodes had distinctly different patterns of labeling. The percentage of medium sized cells labeled tended to be higher in thymus initially, and the labeled cells disappeared more rapidly than in mesenteric nodes. Cervical nodes paralleled mesenteric nodes.

Most of the points for medium sized lymphocytes in figures 1 and 2 are based on the scoring of about 100 cells. To obtain a better picture of the change in grain counts with time, a minimum of 500 medium sized thymus-lymphocytes were scored for the first 24 hours after the injection of thymidine. The data are presented in table 1. It is apparent that there was considerable variability in the grain counts. Presumably at 2 hours, most of the labeling had come from endogenous DNA synthesis, and not from division of larger cells. The variation of grain counts could be due to a combination of factors such as differences in DNA synthesis rates and availability of the tritiated thymidine, as well as the statistics of autoradiography. If no further activity enters a population, the decrease in the mean grain count with time should be a measure of the generation time of dividing cells. Because scattered reticular and large cells, as well as those of germinal centers, were initially highly labeled, the contribution of labeled DNA from the division of these cells to the medium sized class will have to be quantified before the true generation time of medium sized cells can be determined.

TABLE 1 —*Grain Count Distribution of Medium Lymphocytes in Thymus as per cent of Cells Counted*

Grains per cell	Time after injection of tritiated thymidine			
	2 hrs.	6 hrs.	12 hrs.	24 hrs.
>35	0.30	0.48	0	0
31–35	0.59	0.32	0	0
26–30	0.59	1.3	0.80	0
21–25	2.8	1.4	0.60	0.25
16–20	3.6	1.4	2.2	0.62
11–15	12	9.0	8.8	3.6
3–10	39	46	47.9	40
0–2	41	40	39.9	56

Neglecting this at present such a calculation gives a generation time for medium sized cells of approximately one day for thymus and three days for mesenteric nodes. Judging from the mitotic indices reported for rats [9, 16] these times may be too long

The percentage of labeled small lymphocytes initially increased with time in both thymus and nodes The curves are consistent with the generally accepted idea that the small cells are formed by division of larger cells but our in vitro study indicates that some endogenous DNA synthesis also occurs The separation into small and medium sized cells is necessarily arbitrary In general the labeled small lymphocytes had leptochromatic nuclei and were slightly larger than the average of this class but some labeling was observed in smaller cells with distinct chromocenters and pachychromatic nuclei The percentage of labeled small lymphocytes in the thymus was considerably higher than in the nodes and they disappeared more rapidly This rapid disappearance of the highly labeled small lymphocytes presumably indicates either a short time spent by these cells in the thymus or a dilution of the labeled thymidine by mitosis or a combination of both The extent to which thymus lymphocytes contribute to blood and lymph lymphocytes is being studied [18] When more precise data and a good mathematical model are available it should be possible to calculate the rates of formation of small lymphocytes in the different tissues.

The percentages of labeled small and medium lymphocytes from thoracic duct lymph paralleled those from mesenteric nodes fairly closely This suggests that with regard to age distribution the cells released from nodes represent a random sample of what is present in the nodes In general this is in accord with determinations of DNA P^{32} specific activities which were similar for node and thoracic duct lymphocytes [4, 5]

The percentage of labeled lymphocytes in blood resembled that in mesenteric nodes and lymph after the first few hours Figure 3 summarizes the blood data for the first month A considerable fraction of the medium lymphocytes were labeled within a few hours and none were seen after nine days Presumably this is the group of lymphocytes described by Ottesen[11] in humans and by Resegotti[15] in rabbits as having a mean age of three days The percentage of labeled small lymphocytes increased for the first three days and then declined gradually

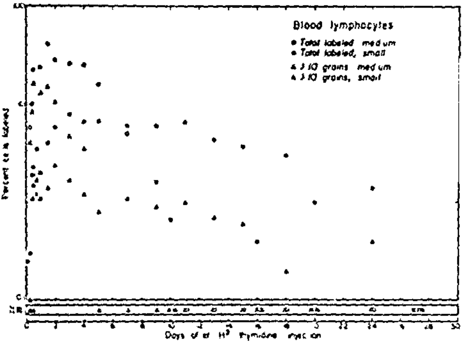

FIG. 3.—Labeling of blood lymphocytes after tritiated thymidine injection. Total labeled cells (>2 grains) and heavily labeled cells (>10 grains) are plotted as per cent of cells in each size category. Instances in which no labeled cells were found (zero labeling) are plotted below the abscissa.

By one month labeled small lymphocytes were rarely seen. The decrease was more rapid than one might have expected from previous estimates made on the basis of DNA specific activities in other species.[18, 7] Although there was considerable variability in the data, the decline in labeled cells appeared to be roughly exponential, with a half time of one to two weeks. It must be noted that this is not a measure of the time spent in the blood but rather a reflection of the time spent in the nodes and possibly the periphery, depending on whether or not one assumes that cells in the periphery are in equilibrium with those in the nodes and blood.

An observation on bone marrow, requiring considerable confirmation but of sufficient interest to warrant mention, was that cells with the morphologic appearance of small lymphocytes (25 to 30 per cent of the nucleated cells) seemed to have a different pattern of labeling than small lymphocytes in blood or lymphoid tissue. The percentage of these cells containing label gradually increased to a maximum of 50 per cent at three days, and then declined. Approximately 10 per cent had more than 10 grains. The identification of lymphocytes in marrow is always difficult, and introduces an uncertainty. However, some confirmation of these results was obtained from an experiment in which multiple daily injections of thymidine were given to mice and rats. Under these conditions, essentially every nucleated cell in the marrow was labeled, whereas only 10 per cent of the lymphocytes in lymph and blood contained label. If these cells are truly small lymphocytes, is this observed difference in labeling related to their ability to act as stem cells in the marrow, as suggested in the past? Theoretically, an accumulation of labeled cells in the marrow would be expected if the lymphocytes enter the marrow in a random manner with regard to age and have a finite life span. Alternatively, this accumulation could be the result of selective sequestration of labeled cells or endogenous DNA synthesis by marrow lymphocytes. These mechanisms considered separately cannot provide a quantitatively satisfactory explanation.

In Vitro Uptake of Labeled Thymidine into Lymphoid Cells

Lymphocytes from rat blood, lymph, and suspensions of thymus and lymph nodes were compared with respect to their ability to synthesize DNA in vitro. Table 2 summarizes the percentage of labeled cells found in the different size classes, and table 3 the differential distribution of these cells. Nearly all labeled cells had high grain counts. Only the thymus showed significant labeling of the small lymphocytes. In the thymus, 2 to 5 per cent were labeled, and the majority of these were in the 7μ range with a leptochromatic nucleus. Because these cells were already well-labeled after 15 minutes of incubation, the label must have come from endogenous DNA synthesis rather than from division of larger labeled cells. These are probably the small lymphocytes that Sainte-Marie and Leblond[16]

TABLE 2.—*Per Cent Labeled Lymphocytes* After in Vitro Incubation with Tritiated Thymidine***

	Incubation Time	Cell diameter in microns		
		5–7μ	8–9μ	≥ 10μ
Blood	15 min	0.05	0	6.6
Thymus		4.4	62	65
Mes. LN		0	3.1	31
Blood	30 min	0	0	1.6
Lymph		0.5	40	60
Thymus		2.2	59	70
Mes. LN		0.2	7.6	66
Blood	1 hr	0.07	2.8	2
Lymph		0.8	83	—
Thymus		5.5	70	87
Mes. LN		0.5	28	74

* Average number of cells scored per sample: 5 to 7μ class, 2000; 8 to 9 class, 150; ≥ 10μ class, 70.

** Cf. Addendum.

TABLE 3.—*Size Distribution of Lymphocytes in in Vitro Incubation Study*

	Cell diameter in microns					
	5	6	7	8	9	≥ 10
Blood	50%	34%	12%	3.0%	1.0%	0.43%
Lymph	90	8.7	1.4	0.32	0.06	0.02
Thymus	82	9.8	4.2	2.4	1.1	1.1
Mes. LN	64	19	13	2.4	0.84	1.1

observed in mitosis. A high percentage of medium and especially large cells were found labeled in thymus, lymph node, and lymph. However, labeling in the medium sized lymphocytes from the mesenteric nodes was considerably lower than in those from thymus and lymph. Since in a short incubation the percentage of labeled cells represents the fraction of the generation time occupied by DNA synthesis, the medium sized lymphocytes from nodes have either a shorter DNA synthesis time or, more probably, a longer generation time than those from thymus.

Blood lymphocytes, when incubated in vitro, showed a much lower percentage of labeled cells than cells from any of the other tissues.* This was found whether the incubation was carried out using whole blood or a suspension of the buffy coat. Red cells did not seem to be responsible for this difference, since thymic cells incorporated thymidine to the same extent whether or not red cells were present. The percentage of cells labeled in blood agrees with that found by Bond et al[2] in human blood. The metabolic difference between the large and medium sized cells in blood and lymph is striking. The larger lymphocytes must come from the lymphoid tissue either by direct entry or via the lymph, where they do have the capacity to synthesize DNA. The fact that those cells found in blood did not incorporate thymidine to any extent in vitro suggests that there might be a selective sequestration of those cells in the process of synthesis upon entry into the blood stream. Alternatively, conditions in the circulation may alter the capacity of these cells to synthesize DNA. In vivo, the percentage of labeled medium lymphocytes in blood was much lower than in lymph at two and six hours, in agreement with the in vitro findings.

Lymphocytopoiesis as Estimated by P^{32} Incorporation

The number of cells formed in lymphoid tissue may be estimated from the rate of DNA synthesis as measured by the incorporation of P^{32} into DNA. In order to determine the fractional DNA turnover accurately, a complete time curve for the immediate precursor specific activity and the DNA specific activity would have to be known. However, as a rough approximation, the DNA specific activity was measured only at two and five hours after the P^{32} injection, and the average specific activity of the acid soluble phosphate was assumed to be the precursor activity. The calculated DNA synthesis rates in mice, together with the total cell numbers, are presented in table 4. The renewal rates obtained were similar to our measurements in guinea pigs[7,9] and rats and to the earlier values of Andreasen and Ottesen[1] and others. Despite all the approximations, it appears that 0.5 per cent to 2 per cent per hour is a reasonable range for the renewal of DNA in rodent lymphoid tissue.

The total number of cells produced, as estimated by this method, may be compared with thoracic duct lymphocyte outputs. In our mice, the thoracic duct output, determined by cannulation in anesthetized mice, was 2 million cells per hour, which is in the same range as the number of cells produced in lymph nodes and thymus. A similar comparison in guinea pigs and rats has led to the same conclusion, namely, that the number of cells produced in the or

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* Cf. Addendum.

TABLE 4.—*Lymphoid Tissue Renewal from DNA P^{32} Specific Activities*

	DNA Specific Activity		Mean Acid Soluble Phosphate Specific Activity	Calculated DNA Renewal	No. of Cells in Organ	Calculated Cell Production per Hour
	2 hrs.	5 hrs.				
Mesenteric and Mediastinal Nodes	4.5	6.3	96	0.62%/hr	1.5 × 10^9	0.94 × 10^6
Cervical and Axillary Nodes	3.6	5.0	45	1.0%/hr	0.63 × 10^8	0.63 × 10^6
Thymus	6.6	10.8	82	1.5%/hr	1.1 × 10^8	1.7 × 10^6

* Specific activities are calculated as counts per minute (cpm) per mg of DNA phosphorus times 100 divided by cpm injected per gm body weight. Mean values from two or three determinations. Male A strain mice, average weight 24 Gm.

ganized lymphoid tissues (excluding the spleen and Peyer's patches) is of the same order of magnitude as the thoracic duct output. It is important to note that the measurement of DNA synthesis rates or mitotic indices gives information on the total number of cells produced. This could be equated with small lymphocyte production only if all the newly formed larger cells eventually differentiated into small lymphocytes. *The fraction of larger cells formed which* leave the nodes or die is not known, but if it is appreciable, it could account for the apparent discrepancy between the high DNA P^{32} renewal rates and the low percentage of thymidine labeled small lymphocytes. Thus, we do not believe that the similarity between thoracic duct outputs and cell production in lymphoid tissue can be used as an argument either for or against lymphocyte recirculation. In addition to the uncertainties in the measurement and interpretation of DNA synthesis rates, the thoracic duct lymphocyte output in the normal animal is not adequately known, since it has been determined in anesthetized or restrained animals. Furthermore, the extent of thymic and splenic contribution to the thoracic duct lymphocyte output, as well as the fraction of lymphocytes produced in lymphoid tissue that enter the blood directly, is unknown.

Osgood[12] has discussed in detail the parameters to be determined before an adequate understanding of leukocytopoiesis and leukokinetics becomes possible. We are confident that autoradiography will eventually allow the determination of many of these parameters, but at present, regrettably, only a semiquantitative picture has been obtained.

ADDENDUM

Additional experiments require revision of the conclusions reached concerning the DNA synthesizing capacity of circulating blood lymphocytes. Recent 1 hour in vitro incubations of whole blood have yielded much higher average percentages of labeled lymphocytes (5–7μ, 0.5 per cent; 8–9μ, 12 per cent; $\geq 10\mu$, 41 per cent) than found in the preliminary experiments reported above. Furthermore, we have found that the presence of red cells in the smears increases the nuclear diameter of the lymphoid cells by approximately 2μ. Thus the more recent experiments largely eliminate the apparent previous difference in labeling between comparable classes of cells in lymph and blood. The results in blood are much more variable than those found in other cell suspensions and may be due to such factors as the level of anesthetics or glucocorticoids in the donor blood

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Some Aspects of Cell Population Kinetics

By HENRY QUASTLER

THE EPITHELIUM lining the small bowel is one of the simplest systems in which cellular proliferation can be studied since about 95 per cent of the epithelial cells are of one type. During its mature life each cell migrates in an orderly fashion from the mouth of a crypt to the tip of a villus.[3] The development of autoradiography with tritiated thymidine[4] has made possible a detailed investigation of the kinetics of cell proliferation and movement in this system. The results have been comprehensively stated elsewhere.[7] In the present paper a few points will be developed which bear on problems of cell proliferation in general.

Both sectioned material and squash preparations can be used for study. The section has the advantage of preserving anatomic relations and thus yielding accurate information on the location of labeled cells. Counts of labeled cells are affected by self absorption and, therefore, are of limited precision; amounts of label in individual cells cannot be estimated at all, but the maximum level of labeling as well as the average level can be estimated with fair precision.

A squash method has been worked out for the intestine by D. R. Wimber at Brookhaven.[8] She isolates individual villi and crypts and spreads all epithelial cells of either structure evenly, with negligible losses. Such preparations yield very accurate determinations of total numbers of cells per villus or crypt, number of labeled cells, and relative amounts of label in individual cells. For the last mentioned purpose, the squash method which reduces all cells to about the same thickness is preferable to the smear.

Combining information from sectioned and squashed material, one can obtain very accurate counts of absolute and relative numbers, which then can be utilized in estimating kinetic parameters within a few per cent. However, it was found that there are considerable variations in the number of cells in individual crypts and villi, in the averages for different mice, in the ratio of villi-to-crypts in different mice, and in the amount of movement of labeled cells onto individual villi within a given time. Since the estimation of the kinetic parameters must be based on combining results from different animals, large numbers of animals would be needed in order to arrive at precise statistics. At this moment it is not planned to collect data on such a scale. The estimates given below are largely based on sections, and are precise to a factor that varies from about 1⅛ for the best estimates to about 2 for the poorest ones.

LABELING INDICES

As the mitotic index is the ratio of cells in mitosis to all cells, so the labeling index is the ratio of labeled cells to all cells. With tritiated thymidine as a label the labeling index is equal, in general, to the fraction of cells that were synthesiz

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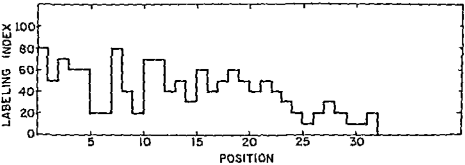

FIG 1.—Labeling index versus position crypt epithelium of lower ileum of 'August' rats

ing DNA at the time of injection. Since the duration of the DNA synthesis phase (S phase) is ≈10 times that of the mitotic phase, the labeling index is correspondingly larger than the mitotic index and therefore easier to determine.

Labeling indices can refer to entire cell populations, or to variously defined subpopulations. The labeling index for the intestinal epithelium, including crypt and villus cells, is 13 per cent (in the lower ileum of a young adult C57 Brown mouse, for other parts of the intestinal tract, and for other strains and species the data are somewhat different).

An example of a labeling index defined with respect to a particular location is given in figure 1. The position of all labeled cells in a number of crypts was noted, the columnar cell bordering on the Paneth cell region being counted as cell No. 1, the cell occupying the next position toward the lumen as cell No. 2, etc.[6] About 50 per cent of all cells in a limited zone are seen to be synthesizing DNA, with a diminution of DNA synthesizing activity toward the mouth of the crypt. A similar situation has been found in various segments of the small bowel of mice and in the cells of the growing hair follicle in mice,[1] the maximum local labeling index usually being between ⅓ and ⅔. This indicates that in the region of maximum synthesizing activity the duration of the S phase is ≈½ of the total proliferative cycle, or more if not all the cells in this region are proliferating.

A labeling index can equally well be defined for a subpopulation of cells in a particular functional phase. Figure 2 shows the labeling index for cells in metaphase or anaphase as a function of interval elapsed between administration of label and sacrifice. This study reveals much about the kinetics of the proliferative cycle. Cells that are found in metaphase or anaphase at the time of examination must have been synchronous to within about ½ hour at the time of sacrifice. Cells found to be labeled must have been in the S phase a few minutes after injection of the label. By varying the interval between injection and sacrifice, it is possible to determine the alternation of S and non S phases preceding the sacrifice.[7] In order to be in mitosis and to be labeled, a cell must have gone at least through phase G_2 and prophase between the time of injection and the time of sacrifice; this is seen to take at least ½ hour. The percentage of labeled mitoses rises to almost 100 per cent over a period of about 2 hours. The length of the rise time is due to three components: variability in the duration of the G_2 phase, variations in the length of time a cell has been in mitosis at the time of sacrifice, and the length of time after injection, during which label was available. It is re-

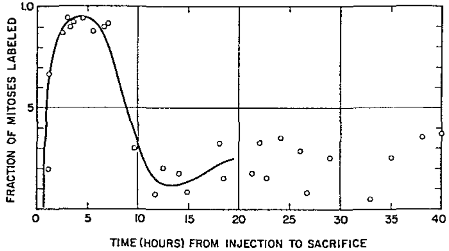

FIG 2 —Percentage of labeled mitoses versus time between injection and sacrifice crypt epithelium of lower ileum of C57 Brown mouse (Reprinted, by permission from Exper Cell Res)

markable that almost all labeled cells seem to go through mitosis soon after termination of the *S* phase, in this tissue, DNA doubling without successive division must normally be rare

A high percentage of labeled mitoses is maintained for several hours, the interval between the two 50 per cent points of the curve being about 7½ hours, this is the mean duration of the *S* phase If the *S* phase were of constant duration, the fall of the curve would be the inverse of its rise Actually it is less steep, which proves that *S* phases in individual cells must be of unequal lengths The low labeling index following the peak is due to cells which were in the G_1 phase at the time of labeling The variability in the duration of the G_1 phase must be very great, this could account for the absence of a well defined peak corresponding to the *S* phase preceding the one observed

The curve shown seems to be characteristic of several tissues similar curves have been found for the bone marrow[2] and for the hair follicle[1]

Over All Kinetics

Assuming that 13 per cent of all intestinal epithelial cells are in *S* phase at any given time, that the *S* phase lasts an average of 7 5 hours and that it is always or almost always followed by mitosis, the hourly production of cells can be computed as 13/7 5 = 1 7 cells per 100 epithelial cells per hour In the steady state, the death rate must have the same value Furthermore since normally all cells go through the phases of the proliferative cycle, G_1, *S* G_2 and mitosis, in that order, and since few of the cells seem to die in the course of normal proliferation, the steady state condition requires that $n_i/t_i = n_s/t_s =$ 1 7 cells per 100 cells per hour, where n_i = percentage of all cells in phase i (i being G_1 *S* etc), and t_i = mean time to go through phase i

The epithelial sheet covering the villi contains about 65 per cent of all cells It is traversed in about 46 hours, as established by serial sacrifice after labeling[7]

If all differentiating cells go through the whole length of the villus then one should find $n_v/t_v = 1.7$ per 100 cells per hour where n_v = the percentage of cells traversing the villus, and t_v = the mean transit time but $65/46 = 1.4$ The discrepancy is not large and is of doubtful statistical significance since the time parameter t_v can be established only with low precision The ratio n_v/t_v might be as small as 1 and as large as 2 This range wide as it is does not allow for heavy mortality of newly produced cells

The analysis of kinetics suggested the existence of a definite transition phase between proliferative crypt cell and mature villus cell For several hours after injection of the label no labeled cells appear on the villi and it takes about 11 hours before the procession of labeled cells moves at full speed This indicates that release from the crypts depends on a labeled cell having gone through mitosis and some subsequent transition phase

J C Hampton of the University of Washington has looked for and identified the transition phase in the electron microscope by a study of the endoplasmic

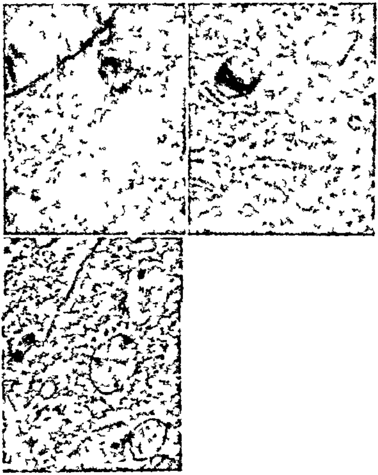

Fig 3—Types of endoplasmic reticulum (a) predominantly vesicular endoplasmic reticulum a proliferative cell (b) tubular endoplasmic reticulum in mature cell (c) [illegible] type in cell near mouth of crypt Enlarged 64 000 times (electron micrographs) (Dr J C Hampton)

reticulum of the cytoplasm[4] Proliferating cells have a vesicular reticulum, mature cells a tubular endoplasmic reticulum Cells that have portions of both kinds of reticulum are found near the mouths of the crypts (fig 3)

CONCLUSION

Even in a relatively simple cell system, various methods had to be combined in order to establish the over all kinetics The most substantial advance was due to the use of cells with two phase markers i e mitosis and labeled DNA It seems that at present, the greatest need in this research is for additional independent phase markers

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Discussion of Results Obtained with Tritiated Thymidine

E L Alpen It is refreshing to hear that with tritium Dr Bond finds the generation time of the orthochromatic normoblast[*] is quite close to what we determined with iron about 20 hours However in reference to his remarks on whether or not the polychromatic normoblast on occasion fails to go through the orthochromatic normoblast compartment or is lost, I think it is worth saying that I feel very strongly on the basis of our iron data that the orthochromatic normoblast is in general a side stream of development of the red cell. It is more or less an accident. If a cell is a reticulocyte at the end of its development it could not have been an orthochromatic normoblast because polychromasia would not have come back in the reticulocyte. So if one assumes that two thirds of the cells that are polychromatic normoblasts then go on to reticulocytes and red cells, this thoroughly explains the observations made by Dr. Bond. We feel that our iron data show this to be the case. Something over one half of the polychromatic normoblasts fail to become orthochromatic normoblasts in the course of their development.

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[*] Some investigators including Drs Bond and Cronkite feel that it is not possible to obtain *constant* and *reproducible* cytoplasmic color reactions when staining through autoradiographic film Accordingly these investigators have used nuclear criteria for identifying cell stages Bond et al define normoblasts in large part on the basis of nuclear diameters admitting however that due to some overlap this alone cannot be used The degree of chromatin condensation presence or absence of nucleoli may also be used Unfortunately in so doing terms which normally are used to describe a tinctorial characteristic of the cytoplasm have been applied to cells which may not have possessed this characteristic Moreover if as Dr Alpen suggests there are two lines of development for the polychromatic normoblast—one in which polychromasia is lost prior to nuclear extrusion (orthrochromatic normoblast) and the other in which the nucleus is extruded prior to loss of polychromasia—then there will be some cells with nuclei of equal diameter and chromatin condensation only one of which has an orthochromatic cytoplasm To clarify this problem these authors have submitted the operational definitions of the cells in question (Bond et al see page 188 Alpen and Cranmore footnote page 290) —*Ed*

V. P. BOND: I hope I made it clear that one interpretation of the curves of labeled erythrocytic precursors would allow for some polychromatic normoblasts to become reticulocytes without going through the stage of the orthochromatic normoblast. This is only one possible explanation. Another explanation of the curves is that the fine structure of the upswinging curve of the polychromatic normoblast may be a stepwise rather than a continuous function. If this is true, and there is no increase in the number of labeled polychromatic normoblasts at the same time that labeled cells are pouring into the orthochromatic compartment, then it is possible to explain the curves without resorting to the loss of cells in the polychromatic compartment.

K. I. FICHTELIUS: Dr. Weicher, in Bonn, introduced a new method of classifying the erythroblast, measuring the diameter of the erythroblast nuclei. He finds that the nucleus of the big erythroblast—which he called the K 2—is twice the size of K 1, and K ½ is half that of K 1, and so forth. He has shown in very small hamsters that the number of chromosomes in K 2 and K 1 when they are dividing is 22; in the last erythroblastic division the number of chromosomes is 12. He has also shown that in the myelocytic stage there is a reduction mitosis to one half.

W. DAMESHEK: I have listened with considerable awe and admiration to these labeling techniques, of which I know less than nothing, and I must say that the tritiated thymidine technic represents a great break-through which I hope will lead to many discoveries. I do hope, however, that this and the other technics will not lead us astray too often. There seems to be some discrepancy in the observations from laboratory to laboratory, particularly with reference to life span of cells and recirculation of blood.

Some observations based on old fashioned morphologic technics may bear on the downslope of Dr. Bond's curve of the granulocytes. The granulocyte in the myelocytic stage, has a large nucleus which becomes progressively smaller as the cell matures. Eventually the nucleus breaks up into lobes which become smaller and smaller, and finally into several small lobes. Is it possible that some of the lobes get lost from the cell, together with DNA?

Lymphocytes are quite different in "nuclear behavior." The medium lymphocyte has a nucleus about the same size as the small lymphocyte. The cytoplasm simply becomes smaller. The small lymphocyte goes along with the same size of nucleus throughout its life span, whatever that span may be.

One wonders about Dr. Hamilton's observations of the very long life span in chronic lymphocytic leukemia. If you look at the blood you see a great many broken down lymphocytes, although their nuclei are more or less intact. These might conceivably exist in the circulation for some months. I wonder if that might account for the observations of very long life spans. When these life spans are measured by isotope technics, one is measuring a certain constituent of a cell, not necessarily the life span of the cell itself.

L. G. LAJTHA: I should like to enlarge on the point mentioned by Dr. Dameshek. The hematologists always can see a decrease in size as myelocytes mature to later forms. In view of this and the differences in grain count which one finds between early and late cells, I think that one ought to view these slopes based on DNA labeling with the utmost caution until one has positive data based on spectrophotometric measurements of the DNA content of cells at various stages.

V. P. BOND: If I interpret Dr. Lajtha's remarks correctly, I would agree to a large extent. Basic to any DNA labeling curve is the assumption that the input data are good i e we can positively identify cells and place them in the correct compartment If we are unable to do this then the resulting curve will not reflect accurately the kinetics of the flow from one compartment to another It is quite possible that morphologic criteria are not good enough for this purpose We are aware that granulocytes decrease in size as they mature and that an hour after labeling the more mature the cell the fewer the grain counts However within one to two generation times the grain counts are high enough to quantify accurately the number of labeled cells With regard to Dr Dameshek s question as to the possibility of pieces of DNA being removed some evidence for this has been presented for the erythrocytic series in bone marrow and may occur also in the myeloid series If this occurs it would of course decrease the grain count over the more mature cells but would not cause the cell to be classified as unlabeled unless the total grain count were very small Such a phenomenon would shorten the time at which an observer could classify these cells as unlabeled and this would probably influence the slope of the curve only to a small degree

F Stohlman Jr As Dr Bond mentioned labeling with tritiated thymidine is not a tracer type of experiment in the usual sense of the word All cells are not labeled but only those in DNA synthesis I assume the labeled cells on the average will divide at a different time than the unlabeled cells Consequently one might anticipate that there would be waves of labeled and unlabeled cells which would gradually be integrated if one contends as I do that these are not like marching soldiers The waves may or may not be distinct but this uneven labeling among the precursor cells might affect the downgrade of the curve

Secondly if one envisions several divisions between the precursor cell and the final end product and death of cells occurs at the myeloblast stage the number of divisions must be increased several fold over what would be needed if a metamyelocyte died There may be only 1 or 2 per cent of the total marrow cells dying If these are all mature cell the death process is not very significant On the other hand if death occurs amongst the erythroblasts or myeloblasts then the number of divisions by the primitive proliferative cells must be increased substantially and the rate of dilution of the label will be much more rapid Thus of two neutrophils entering the peripheral blood one may be a first generation neutrophil and the other a second or third generation cell

H M Patt In reply to Dr Stohlman s comments there is a suggestion of a cyclic pattern of increase and decrease of labeled neutrophils in the two dogs that have been studied at frequent intervals during the third and fourth days after H^3 thymidine injection The peaks are separated by 15 to 20 hours which is in good agreement with the approximate mean generation time of myelocytes These findings are receiving further attention in present experiments

N B Everett In reference to Dr Kelly's work I would like to mention another thing which I think is significant In plotting out the percentage of labeled cells in hours rather than in days we noted that the labeled large lymphocytes reach a peak relatively soon Then we get a cycling of the labeled cells the large lymphocytes first and with time the peaks getting higher The medium lymphocytes behave similarly but their peak comes later Then the small lymphocytes peak comes later still This same cycling continues for many days The intensity of labeling is less with time which I think is very significant Also we must consider the degree of reduction in our emulsion since the grain counts will become more significant in interpreting the data I would like to cite one example A maximum of about 7 per cent of the lymphocytes are labeled one day after thymidine One month after thymidine in a single guinea pig we found about 2 per cent of the lymphocytes labeled However the intensity of labeling was only a fraction of that seen in the same cell type at earlier stages Only occasionally do we bump into a lymphocyte of the small variety that has more than a moderate labeling at the end of a month

T E Harris I was interested in Dr Bond s curves because the increase and then decrease of the number of labeled cells seems similar qualitatively at least to the situation that is observed for certain types of particles that occur in the nucleon com ponents of cosmic ray showers To the extent that the cell phenomenon is due to the dilution effect caused by division of cells it seems to me that the mechanism is somewhat similar that is in the case of the cosmic rays a particle which has some energy has a collision and causes two new particles to arise, so we might as well say that the original particle is divided in two The energy of the original particle is shared between the two new ones which then go on to share their energy among more particles and so on If we plot the number of particles having energy greater than some fixed level we get this ascending and descending curve I gather that the dilution effect is not adequate to explain entirely the curve in the biologic situation but perhaps it would be of some interest to apply the methods used by physicists for the cosmic ray case to see just how much of the effect one could attribute to dilution

H Eyring If about half of the cells are labeled, and then about 20 hours later you get a wave of unlabeled cells you must assume that these cells can be labeled during only about half their lifetime then they divide, and each division gives you a new wave of labeled cells Is this not correct?

V P Bond I think this depends a great deal on the degree of equivivancy in the population i e what sort of time distribution you get in time how nearly do the cells stay in step Dr Patt apparently has observed these waves in the peripheral blood We have looked very carefully but have not observed the phenomenon However it is quite possible that we have not taken samples with sufficient frequency

H Quastler In good squash preparations the number of grains over a given cell should be reasonably proportional to the amount of label in this cell Therefore frequency distributions for grain counts are fairly representative of frequency distributions of the amount of label D Wimber at Brookhaven has obtained such distributions for crypt cells She finds a distribution with two well separated peaks about one order of magnitude apart The frequencies of cells with 1 2 3 4 and 5 grains fit a Poisson distribution One might assume that those few grains are due to background However this assumption does not account for the large percentage of cells with no grains at all (even if one assumes that all heavily labeled cells could contribute to the 1–5 grain categories) Hence we suspect that these cells with a few grains are actually labeled We do not know however whether the label is in DNA and if so whether or not it is associated with DNA metabolism not linked to cell division

Dr Bond s interpretation of the steepest part of the upswing of his curve holds only under steady state conditions It does not hold if there are variations in the rate of delivery of labeled cells into the blood Such variations could be due to diurnal variations in the release of cells in general or to a mixing pool in the bone marrow from which mature cells are released If such variations exist Dr Bond s method of checking for appearance of labeled cells in the periphery is bound to pick them up The trouble is that such an effect would yield an overestimate of the steady state rate of entry of labeled cells and thus invalidate the interpretation that the steepest part of the curve is necessarily a lower bound of this rate

V P Bond I think that the upper limit (longest time *Ed*) for the turnover time of blood elements derived from our analysis is independent of the considerations Dr Quastler mentioned provided that the system is reasonably close to being in a steady state If we treat tritium labeled thymidine as a tracer and labeled cells enter a compartment at a certain rate this must represent an upper limit for the rate (slowest rate *Ed*) of movement of all cells into this compartment Cells must be entering at least at this rate and in the steady state must be leaving at a similar rate The turnover time we derive from this is an upper limit the true value could be considerably less Three independent groups have obtained a similar curve for the appearance of labeled granulocytes in the peripheral blood in three different species It is possible that a steady state as we usually think of it does not apply and that the production of cells instead of being a continuous process occurs only at intervals perhaps at irregular intervals There may be a diurnal variation The fact that the same curve has been obtained under different conditions leads me to believe that if there is such an effect it is minimal and that the values we derive probably represent a value very close to the upper limit for the turnover times for the cell types considered

H Von Foerster As Dr Quastler has pointed out if one drops the assumption that all cells behave exactly alike the expected output is no longer a sharply defined step-function but may assume the shape of a smooth curve This shape is defined not only by some mean values of certain characteristics of these cells but also by the way these characteristics are distributed amongst the cells By assuming different distribution functions the shapes of the resulting curves may change drastically Since we do not have any particular idea about the processes which are responsible for the variations of our characteristics we are pretty much in the dark with respect to the choice of a distribution function normal log normal linear etc By a lucky choice of a particular distribution function we may obtain a good agreement with data obtained from a particular experiment The question which remains to be answered is of course Do we now adequately describe the facts or are we just skillful in juggling up flexible equations which happen to fit our points? In this context I would like to point out that in many instances we can observe only very small samples of cell populations Since it is not too absurd to believe that the microactivity of the whole organism is not rigidly synchronized it would not surprise me if in a single experiment some unexpected local burst in productivity produced little humps in the output curves Probably these humps would straighten out if hundreds of such experiments were carried out using of course exactly the same rats—if one could get them

Section IV. Studies on Leukocyte and Platelet Production

L. M. Tocantins, *Chairman*

Circulation Time and Fate of Naturally (Alkaline Phosphatase) and Fluorescent (Atabrine) Labeled Granulocytes

By George Brecher and James C. Peden, Jr.

FROM THE SUCCESS of the transfusion technics in the measurement of red cell life span one may justifiably generalize that the life span of a given cell type can be readily established by transfusion of viable and identifiable cells into a compatible recipient. Unfortunately, viability and compatibility of blood cells are best defined in terms of normal survival in the circulation. For measurement of red cell survival by transfusion this is no handicap because the isotopic measurements of Shemin and Rittenberg have established the normal red cell life span quite independently of transfusion experiments. In contrast, all attempts to date at measuring white cell life span by transfusion suffer from our inability to be certain of normal viability of cells and compatibility of donor and recipient in the absence of prior knowledge of white cell life span. However, since incompatibility and reduced viability can be presumed to shorten cell life span, any results from white cell transfusion experiments may at least be considered as minimum periods of normal leukocyte life span.

The classical method of identifying donor cells in the recipient, Ashby's method of differential agglutination, is inapplicable because of the impossibility of identifying blood group antigens in white cells by currently available methods. *Therefore, some other method of identification of donor cells or artificial labeling* of the donor cells must be used. Differentiation of granulocytic donor cells by their content of alkaline phosphatase and atabrine labeling of white cells will be briefly reviewed as examples.

Atabrine (quinacrine hydrochloride) has been reported as a satisfactory label for granulocytes by White[1] and by Lissac and associates.[2] These authors claimed that the white cells are made permanently fluorescent by in vitro and in vivo exposure to atabrine and that the tag is not removed by repeated washing or lost from circulating cells. The circulation time of tagged white cells was reported as 40 to 90 minutes in both rabbit and man, whether in vitro or in vivo labeling was employed. The similarity of curves after in vivo and in vitro labeling ap-

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From the National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Md.

peared to exclude major damage to the cells by the process of transfusion itself. Sequestration of many transfused cells in the pulmonary circulation was reported with subsequent shift of these cells from the lung to the spleen, where they reached a maximum at 4 hours. Because the total life span of only 90 minutes appeared unlikely to Lissac in view of available isotopic data, he concluded that these represent circulation times and postulated that the cells must be in equilibrium with a large extravascular pool. After dilution in this pool estimated at 50 to 100 times the number of circulating white cells, the chance of spotting labeled cells among those re-entering the circulation would be only 2 per cent even though all circulating cells were originally labeled. It occurred to us that repeated injection of atabrine, say in one hour intervals, should allow us to label successive crops of white cells re-entering the circulation. Accumulation of atabrine in the plasma was not expected because Lissac indicated that complete plasma clearance occurred in 10 minutes. It was considered that a readily measurable level of labeled cells could be achieved after 10 injections even if the pool was 100 times the size of the circulating white cell compartment.

Our inability to reproduce the data of White and of Lissac et al. on normal cells (table 1) was disappointing. We confirmed that very heavily labeled cells can be washed three times without any *apparent* loss of fluorescence. However, with additional washing or initial lighter labeling of the cells, a loss of label is readily seen. The supernatant from the third wash still retains the ability to label fresh cells even though the original label was quite light. Addition of fresh cells to thrice washed labeled cells results in transfer of the label to the fresh cells in 5 to 15 minutes, depending on the intensity of the original label.

In our experience and in that of Kosenow[3] atabrine principally labels the granules in the neutrophil, although we cannot exclude a much lighter labeling of the nucleus. We could not confirm that atabrine is primarily a nuclear label as claimed by others. This last point is particularly perturbing since it would suggest that our technic has been at variance with that of others. Fortunately, through the courtesy of Dr. White we were able to obtain a sample of the atabrine used in his studies. Its spectral absorption curve and behavior in in

TABLE 1

	White	Lissac et al.	Brecher and Peden
Location of label	Nuclear	Nuclear	Cytoplasmic
Stability of label in vitro			
Exchange with unlabeled cells	Non exchangeable	Non exchangeable	Slow exchange
Effect of washing	Not removed by washing	Not removed by washing	Removed by repeated washings
Loss of label in vivo after injection	Loss of *labeled cells* over period of several hours	Loss of *labeled cells* over period of 3–40 minutes	*Dilution of label* over period of several hours
Suitability for labeling			
In vitro	Yes	Yes	No
In vivo	—	Yes	No

vitro and in vivo experiments were identical with the atabrine generously supplied to us by Winthrop Laboratories The latter was in the form of a sterile powder and was used for an in vivo experiment in one of us Following the intravenous injection of 100 mg of atabrine, all circulating granulocytes were labeled The intensity of the label gradually diminished over several hours At the end of four hour , 100 per cent of the granulocytes were still labeled, though rather weakly so Continued exposure to the ultraviolet light would blanch the fluorescence in some cells so that in any given field a varying percentage of apparently labeled and unlabeled cells could be artificially produced

We must, for our part regretfully dismiss the fluorescent labeling technic with atabrine because of the gradual loss of label—quite apart from the subsequent blanching under direct observation in UV light which we only mentioned because it might conceivably account in part for the results of Lissac and of White As the label fades longer observation of each field in the UV may be needed for identification, thereby blanching individual cells and artifically inducing an appearance of increasing numbers of unlabeled cells

The use of alkaline phosphatase as a natural label was previously reported by us [4] Rat granulocytes which contain alkaline phosphatase, were transfused into mice whose white cells are devoid of this enzyme The experiments were undertaken not so much in the hope of establishing normal life span, since the donor and recipient were clearly incompatible, but rather in the hope of tracing in tissue sections the large number of granulocytes that never circulate, as indicated by previous transfusion experiments in dogs [5]

By using the WBC s of several large rats for a single mouse experiment, the recipient's count was elevated temporarily up to 40 000 per cu mm Even this high level of circulating cells however, represented only 20 per cent of the total cells transfused Of this 20 per cent, a high proportion circulated for several hours, and a few for as long as a day (fig 1) Sequestration in the lung during the

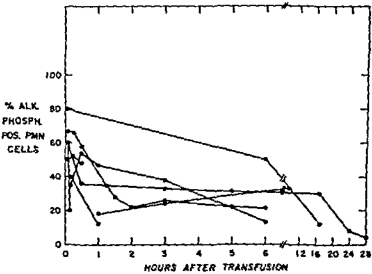

FIG 1 —Survival of rat granulocytes following infusion into mice Survival expressed as per cent of injected cells (positive alk phosphatase) (Reprinted from Brecher et al Problems of leucocyte transfusions Acta haemat 20 1*9 1958)

first hour was evident from the large number of alkaline phosphatase positive cells in capillaries and major vessels After two hours, the pulmonary accumulation of positive cells had disappeared, and only a few cells could be identified in liver and spleen In the liver the few identifiable rat cells were clearly intravascular In the spleen their number was equally low, though intravascular and extravascular locations could not be differentiated with certainty In the other tissues, including lymph nodes, alkaline phosphatase positive granulocytes were extremely rare Contrasting this to the early accumulation in the lung, and considering that the total count in the periphery continued to drop the conclusion appears warranted that huge numbers of transfused cells are destroyed without a trace Whether this destruction occurs intravascularly or not is impossible to say We are inclined to think, however, that cells leaving the circulation are immediately destroyed since we could never identify alkaline phosphatase positive cells extravascularly The single exception to this was found in a peritoneal exudate, artificially induced by introduction of large volumes of nutrient broth A small number of alkaline phosphatase positive cells appeared in the exudate These cells were capable of starch phagocytosis The percentage of these cells was always smaller than that in the peripheral blood suggesting that emigration from the blood is not random or that the rat cells are shorter lived than the mouse cells, once they leave the circulation Conceivably new cells freshly released from the marrow are the first to go to sites of infection as suggested by Fleck[6]

In summary, the fluorescent label, in our studies has proved an inadequate tag The naturally labeled rat cells survived in mice for up to 24 hours On infusion of huge numbers of these cells, only a small fraction could be traced It is suggested that the body has a great ability to destroy white cells particularly extravascularly, with the exception of sites of inflammation The limited scope of incompatible white cell transfusion is stressed

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An Analysis of Leukocyte Radioactivity Curves Obtained with Radioactive Diisopropylfluorophosphate (DFP^{32})

By A. M. Mauer, J. W. Athens, H. R. Warner, Helen Ashenbrucker, G. E. Cartwright and M. M. Wintrobe

THE REACTION of diisopropylfluorophosphate (DFP) with the amino acid group that forms the reactive center for several enzymes is shown in figure 1. The DFP molecule forms an irreversible bond with these enzymes, releasing HF and yielding a diisopropylphosphate-enzyme molecule. When the enzyme is broken down, diisopropylphosphate (DIP) is released. DIP is nonreactive and is promptly excreted.

The irreversibility of the bond and the lack of reutilization of the breakdown product suggested the use of this molecule, when tagged with P^{32}, as a cell label.[2] We have been concerned with the use of DFP^{32} as a granulocyte label. This can be done if certain criteria are met. First, since the enzymes that are bound by DFP are present in red cells, plasma and platelets, isolation of the leukocytes must be complete. Second, of the leukocytes in the blood, only granulocytes must label to a significant degree. Finally, there should be no measurable effect of the DFP on granulocyte function in the concentration used to label the cells. The evidence that these criteria have been fulfilled has been presented.[1]

Granulocytes have been labeled both in vivo and in vitro by DFP^{32}. In vivo labeling has been done by the intravenous injection of DFP^{32}. Granulocytes are labeled in vitro by drawing whole blood into plastic bags with ACD solution as an anticoagulant and incubating the blood for one hour with DFP^{32}. The labeled blood is then returned to the subject. The methods for isolation of the leukocytes and determination of their radioactivity have been described.[1]

A representative leukocyte radioactivity curve obtained after the intravenous injection of DFP^{32} is shown in figure 2. The lower curve represents the radioactivity as CPM per mg. of leukocyte nitrogen. The upper curve is drawn through the logarithms of the leukocyte radioactivities.

During the first two days after the injection of the DFP^{32}, there is a rapid decrease in leukocyte radioactivity (phase I). The leukocyte radioactivity then remains fairly constant until the 11th day (phase II). A final decrease in radioactivity (phase III) follows. By about the 24th day, no radioactivity can be measured in the leukocyte samples.

The results obtained in two subjects after in vitro labeling of their granulocytes are shown in the lower portion of figure 3. The leukocyte radioactivities are expressed as the logarithms of the CPM per mg. of nitrogen. For comparison, in the upper portion of this figure the logarithms of the leukocyte radioactivities obtained during phase I, after in vivo labeling of leukocytes in two subjects, are

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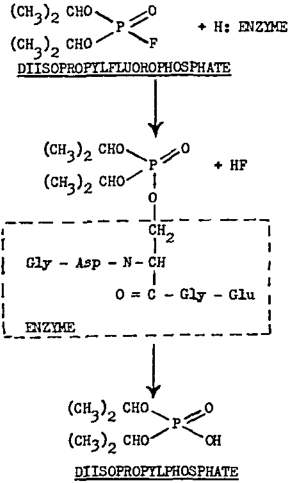

FIG 1 Model of the reaction of DFP with the active serine group of an enzyme Gly Asp and Glu refer to the amino acids glycine aspartic acid and glutamic acid (from Athens et al Blood *14* 303, 1959)

shown The open circles are the logarithms of the values obtained by subtracting the mean phase II values from phase I

If the curve obtained after in vitro labeling is compared with the curve obtained after in vivo labeling a clear difference can be seen Analysis of the curves makes it appear likely that granulocytes in sites other than the blood are being labeled by the in vivo method

To assist in the analysis of the curves, kinetic models were set up on an analog computor and tested for feasibility The first step was to define the possible types of cell compartments that might be present in any granulocyte kinetic model These types are represented in figure 4 In this figure the cells entering the compartments are considered to be unlabeled whereas those in the cell compartments are considered to be labeled maximally at zero time

A type I compartment is a simple, miscible pool of cells that leave in a random fashion The radioactivity expected in the cells coming from this pool after time t_0 would reflect the average radioactivity of the pool itself and would decrease

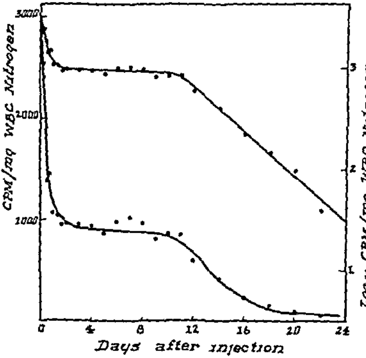

Fig 2 — Arithmetic and semilogarithmic curves of the leukocyte radioactivities obtained after the intravenous injection of DFP^{32} Each curve represents the mean of seven individual curves (From Athens et al Ann N Y Acad Sc in press)

TABLE 1 —*Comparison of the In Vivo Phase I with the Disappearance of In Vitro Labeled Cells*

Groups	No Subjects	Mean T½ ± S.E. (Hours)	P
In Vivo	30	6.4 ± 0.37	>0.05
In Vitro	20	7.4 ± 0.33	

in an exponential manner The mathematical expression of this decrease is given below the model

A type II compartment would be expected if cells in a particular phase must spend a definite time in the phase before release Thus, the cells would behave as if they were coming down a tube aging as they proceed pushing before them older cells and being replaced by younger cells Once the cells in the tube had been labeled, there would be no change in the radioactivity of cells leaving the tube, until the youngest cells at the time of labeling had left and were replaced by cells that were not in the tube at the time of labeling At this time, the radioactivity of the cells leaving the tube would suddenly fall to zero The mathematical expression of the radioactivity of the cells leaving the tube is given below this model Time t_1 is the time when the last labeled cells leave the tube From t_0 to t_1 is the transit time through the tube A sample of blood drawn for analysis at any time would reflect the average concentration of label in the pool and would decrease linearly with time

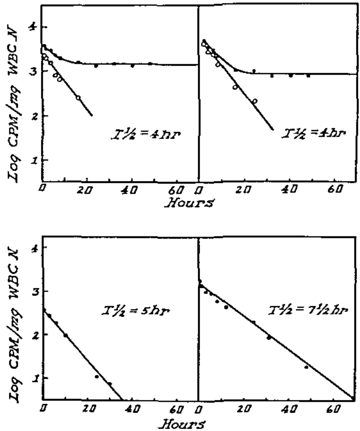

FIG 3 —Comparison of the in vivo phase I curves (*top*) with the disappearance of in vitro labeled granulocytes

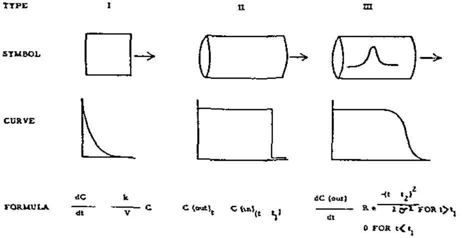
TYPE
I
II
III
SYMBOL
CURVE
FORMULA
dC/dt k/V C
C (out)$_t$ C (in)$_{(t - t_1)}$
dC (out)/dt R e^{-(t - t_2)^2 / 2σ^2} FOR t>t$_1$
0 FOR t<t$_1$

FIG 4 —Types of cell compartments that may be present in a granulocyte kinetic model

The type III compartment is similar to the type II compartment, except that there is some degree of random distribution of the transit time through the tube Thus, after the cells in the tube had been labeled, no change in the radioactivity of the cells leaving the tube would be expected until time t_1, the minimum tran it time. The rate of decrease would become maximal at time t_2, when most of the labeled cells were being replaced by unlabeled cells. Some radioactivity would remain in the cells coming out after this time because of mixing of lagging labeled cells with the unlabeled cells. The mathematical expression of the radioactivity of cells coming from this pool is given below the model. R is the maximal rate of change of radioactivity in the emerging cells and occurs at time t_2.

From the radioactivity curves obtained after labeling granulocytes with DFP^{32} in vitro, certain deductions concerning the removal of granulocytes from blood can be made. If granulocytes leave the blood in a random fashion, the blood pool of granulocytes would represent a type I compartment. The fact that the time course of in vitro labeled cells falls exponentially argues for the validity of this concept (fig. 3). If, on the other hand, granulocytes leave the blood compartment in a sequence determined by their time of entry, then the blood compartment would be type II. Since this model would predict a linear decrease in labeled cells, it can be discarded as not compatible with our data. However, the possibility must also be considered that granulocytes are removed from the blood by a combination of these two methods of removal. To investigate this possibility, three such models have been analyzed with the analog computor, but will not be described here. Observations made from these models suggest to us that granulocytes are removed from the blood in a random fashion, entirely or predominantly, independently of the length of time that the cells have circulated in the blood.

From the curves obtained after the in vitro labeling of granulocytes, information concerning the return of granulocytes from the tissues to the blood can be obtained. The model that is shown in figure 5 was subjected to computor analysis. At a rate of return equal to one half the rate of removal from the blood ($k_2 = \frac{1}{2} k_1$) the lower limits of the size of the tissue pool could be defined. Fair approximation of the data and computor curves were obtained when V_T was allowed to be *greater than* 2.6 times the size of the blood pool (V_B). Upper limits for the return

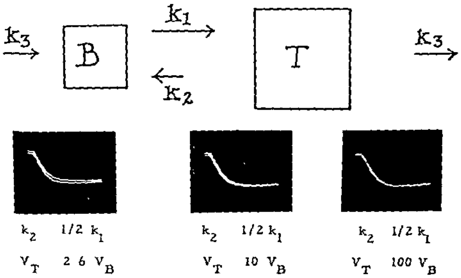

FIG. 5.—Model for possible return of granulocytes from tissues. The method by which the limits can be set for the size of V_T for any k_2 is shown below.

of cells from tissues can be defined by analysis of the in vivo curves as will be described

The curves obtained after the in vitro labeling of granulocytes are also of assistance in the analysis and interpretation of the more complex curves obtained following the in vivo labeling of granulocytes. As shown in figure 3 and table 1 the mean half life of the phase I portion of the curves obtained after in vivo labeling and the half time of disappearance of in vitro labeled cells are similar. This suggests that phase I of the in vivo curve is due to the removal of granulocytes from the blood. This explanation could be valid if it were assumed that the cells in the blood at the time of the intravenous injection of DFP^{32} are labeled to a greater degree than the cells in the marrow and tissues. This assumption would seem to be resonable since it would be expected that the circulating cells would be exposed to a higher initial concentration of DFP^{32}. Therefore in the analysis of the in vivo curves in the analog computor we have assumed that phase I is explained by the removal of highly labeled cells from the blood compartment. On this assumption the initial relative radioactivity of blood granulocyte has been given a value of 100. The radioactivity of the cells in all other compartments has been set at 30. This value was selected because the granulocyte radioactivity levels during phase II are about 30 per cent of the initial value of phase I. Models composed of several postulated anatomic and kinetic granulocyte compartments were then constructed and submitted to analysis in the analog computor. A composite model is shown in figure 6

M_1 is the pool of mitotically active cells in the marrow. The assumption has been made that the label in this pool decreases in an exponential manner because of cell division and that the level of radioactivity in the cells leaving this pool reflects the radioactivity of the pool. Therefore this is considered a type I compartment. Cells leaving the mitotic pool (M_1) enter a maturation phase (M_2) from which they normally would not be released until maturation had been completed. M_2 is thus a type II compartment. M_3 represents a storage pool of mature granulocytes in the bone marrow which is ready for release into the blood. The cells contained in this pool all being mature are thought to be released in a random fashion representing a type I compartment. B is the granulocyte pool in the blood. The small X compartment in equilibrium with the blood acknowledges the nonhomogeneous nature of the blood pool. If equilibra

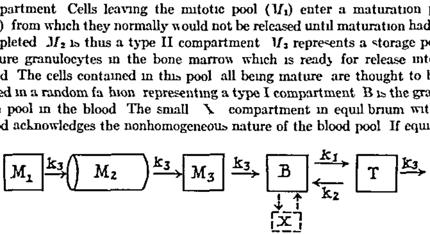

FIG 6—A composite granulocyte kinetic model

1 V_{M_2} is equivalent to V_{M_3} in its effect on the shape of the curve

2 With M and M_3 present only the sum of V_{M_1} and V_{M_3} influence the curve

3 The upper limit of V_M and V_{M_3} is 10 V_B if k_2 0

4 The in vitro curve and phase I of the in vivo curve define the lower limit of V_T for a given k_2

5 The upper limit of V_T is defined by phase III of the in vivo curve for a given k_2

6 As V_T increases V_{M_1} and V_{M_3} must decrease in size

tion were rapid between cells in the circulating blood and the marginal population of granulocytes described by Vejlens,[6] as well as with other possible intravascular pools in the spleen, lungs, etc., the main effect of this "X" pool would be to lead to underestimation of the size of the population of blood granulocytes, since only blood volume and granulocyte concentration are measured. Finally, *T* is the possible tissue pool of granulocytes that may be in equilibrium with blood granulocytes.

By setting up variations of this basic model on the analog computer and comparing the results with the *in vivo* data curve, certain generalizations have been made concerning the nature of this granulocyte kinetic model. These generalizations are given in figure 6. Needless to say, the validity of the model depends upon the validity of the basic assumptions already stated.

One of the most interesting observations drawn from this analysis is the limit that can be set on the amount of granulocyte return from tissues to blood. Using analysis of the *in vitro* curve and the *in vivo* curve to set limits for the size of the tissue pool and the rate of return, as expressed in generalizations 4 and 5 of figure 6, a maximum return rate amounting to 0.3 of the removal rate is found to be compatible with the data curves.

If a type III compartment replaces the type II compartment that was used to represent the marrow maturation phase in the previous model, a fit can be obtained with the data curve, even if no labeling of the mitotic pool is allowed and the marrow storage pool is disregarded. The standard deviation of random distribution about the mean transit time through the compartment had to be two days. This model is shown in figure 7. The bottom curve represents the random distribution of transit time through the compartment. The middle curve is obtained from the computor model, and upper curve is the data curve.

So far it has not been possible to construct a more exact granulocyte kinetic model from the DFP[32] data than that presented in figure 6, because of lack of information concerning the degree and extent of marrow labeling. In the near future, it is planned to use tritium labeled DFP. Demonstrations of the exact distribution of DFP within the granulocyte series should be possible with radioautography of marrow and blood samples. With this information, elimina-

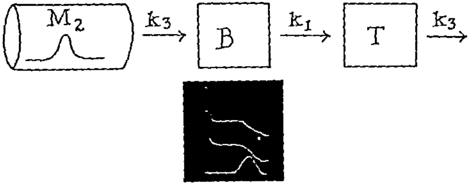

FIG. 7.—A type of granulocyte kinetic model (with type III compartment) with random distribution in the maturation phase. The computor model curve is compared with the *in vivo* data curve. The standard deviation of random distribution was set at two days.

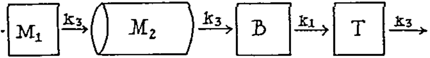

FIG 8.—The simplest granulocyte kinetic model (model 'T') by which the analog computor curve can be made to approximate the in vivo data curve

1. $-\frac{d C_{M_1}}{dt} = \frac{k_3 C_{M_1}}{V_{M_1}}$

2. C_{M_2} (out) $= C_{M_2}$ (initial) for $t < t_1$

3. C_{M_2} (out)$_t = C_{M_2}$ (in)$_{(t-t_1)}$

4. $-\frac{d C_B}{dt} = \frac{k_3}{V_B} [C_B - C_{M_2} \text{ (out)}]$

tion of several of the present models should be possible, and a closer approximation of the actual granulocyte kinetic model may result

With granulocyte kinetic models, calculations of certain kinetic data can be made To demonstrate the type of information that can be gained from analysis of the DFP^{32} curve by means of a kinetic model, the simplest workable model will be used as an example This model, with its defining formulae is shown in figure 8 The designation 'T' is employed to suggest that just as with the early Ford cars, when more information about the mechanism is obtained, more and better models will follow

In the analysis of the DFP^{32} in vivo data curve by this model, phase I represents the removal of blood granulocytes, and because no return of granulocytes from tissues is allowed, this is equal to granulocyte turnover The granulocyte turnover, in turn, represents production in the steady state Phase II is the time necessary for transit through the maturation tube The shape of the curve in phase III is influenced in this model by the rate of granulocyte turnover through blood and the decrease in radioactivity because of cell division in the mitotic

TABLE 2.—*Results of Analysis of Model T*

Phase I

$T\frac{1}{2}$ = 6.4 hours
Granulocyte turnover time in blood = 9.4 hours
Number of times the blood granulocyte population is turned over per day = 2.5
Calculated number of blood granulocytes = 25 × 10^9
Number of granulocytes turned over per day = 62.5 × 10^9
% of blood granulocytes turned over per hour = 10%

Phase II

Duration = 11 days
Time spent in maturation tube = 11 days
Size of maturation tube = 27.5 × blood granulocytes = 687.5 × 10^9

Phase III

$T\frac{1}{2}$ = 3 days
Generation time of mitotic pool = 3 days
Size of mitotic pool = 7.5 × blood granulocytes = 187.5 × 10^9

TABLE 3 —*Comparison of Granulocyte Kinetic Estimates*
(Expressed as Cells × 10^{-9}/Kg Body Wt)

	Cells Produced Per Day	Total Marrow Population	Mitotic Pool
Osgood		25 7	4 3
Patt	2 6	8 3	1 4
Donohue et al		11 4	2 6
DFP^{32} (Model T)	0 9	12 5	2 7

pool Because of the rapidity of cell turnover through blood, the slope of the line is influenced primarily by the effect of cell division in the mitotic pool Thus, assuming phase III to be a simple exponential decrease, by measuring the half-time, one obtains a fair approximation in this model of the doubling time in the mitotic pool

With this model, the kinetic calculations presented in table 2 were obtained Comparisons of these calculations with those of Osgood,[4] Patt,[5] and Donohue et al [3] are shown in table 3

Obviously more work is required to define more exactly the granulocyte kinetic model and its relationship to the DFP^{32} data However, as demonstrated by the analysis of the simple model presented above, one may expect to obtain valuable data concerning granulocyte kinetics in normal and pathologic conditions when these definitions have been established

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Discussion of Data Obtained with DFP^{32}-Labeled Granulocytes

G E CARTWRIGHT DFP^{32} studies have the advantage that they can be done in normal human subjects. Less than 200 µc of P^{32} radioactivity are required Twenty ml samples of blood from subjects with a normal total leukocyte count are adequate to determine radioactivity and total nitrogen content With the simple plastic scintillation counting method which Drs Athens and Mauer have developed it is possible to work with an efficiency of 75 per cent for phosphorus There are problems that have not been mentioned Red cells label more highly than do leukocytes Platelets label as well, and the red cells and the platelets must be separated completely from the leukocytes Dr Athens developed the combination dextran lysolecithin method for making this separation and we have detailed studies indicating that there is less than 2 per cent contamination with radioactivity from red cells and platelets Secondly we have been unable to *uncover any evidence to indicate that there* is elution of label from the granulocytes This of course is not to say that it does not elute Another consideration is that the label damages the granulocytes The phagocytic activity motility and respiration of leukocytes exposed to wide ranges of DFP^{32} concentration both in vivo and in vitro have been unaltered

It is indeed fortunate for us I believe that the lymphocyte does not label significantly with DFP The amount of radioactivity in lymphocytes is insufficient to influence the granulocyte curves

I would like to emphasize that following in vitro labeling these cells are removed randomly and that the rate of removal is rather rapid The similarity between the rates of removal after in vivo or in vitro labeling would seem to argue against the possibility that the granulocytes have been damaged during the process of handling Indeed we have handled them not at all *except to draw them into a plastic bag* put the DFP^{32} in the bag and then retransfuse

Finally if there is no return of leukocytes from the tissues we have no method available for studying the size of the tissue pool No matter what label one uses if there is no return from the tissues then that compartment is really outside of the model being studied Accordingly the best we could do with the help of Dr Homer Warner is to set limits on this possibility assuming return and no return

Perhaps I should say that our model T should not be taken seriously It is of course, our most modern model but I think it is fair to tell you that the survival times of the many models which preceded it were rather short

F Stohlman Jr. With the apparent differences in the blood transit times obtained with thymidine as compared to DFP^{32} the problem of elution becomes important We and others have studied red cell survival using DFP^{32} as a label In our studies in dogs we observed a biphasic curve with an initial rapid disappearance of radioactivity similar to that observed by Dr Mauer in granulocytes We had similar results when we labeled cells in vitro When the amount of DFP administered (in vivo or in vitro) was less than 0 1 γ per cc of red cells the initial fast component of the *curve was not seen*

Using a double label DFP^{32} and Cr^{51} it was observed that cell survival measured by Cr^{51} was normal but the DFP was lost We concluded that the label was eluting rather than that cells were dying In view of this experience together with the discrepancy between H^3 and DFP^{32} granulocyte data might it not be worthwhile to cite your evidence against elution so that each person might evaluate it for himself?

A M Mauer Obviously the problem of elution is important In our procedure the leukocyte button, obtained after dextran sedimentation and hemolysis of red cells with lysolecithin is washed four times in cold isotonic saline If there is elutible DFP in the leukocyte it is probably removed at this stage In any case further washing of the leukocytes does not remove any more of the radioactivity When isolated purified leukocyte samples were treated with trichloracetic acid all of the *radioactivity was recovered in the protein* precipitate Labeled leukocytes have been incubated in vitro and insignificant amounts of radioactivity have been recovered in the suspending media

In addition to the enzymes in the red cell which irreversibly bind DFP another enzyme fluorophosphatase has been described which hydrolyzes DFP to DIP The loss of DFP^{32} from red cells may not be due to elution from the irreversibly bound enzymes but to hydrolysis of the compound by fluorophosphatase

When whole blood is labeled in vitro and then transfused back into the donor the radioactivity in the leukocytes isolated from the experimental subject decreases in an exponential fashion If both elution and removal of labeled granulocytes were responsible for the decrease in radioactivity a biphasic curve such as you have described for red cells might be expected This is not the case Thus by several different types of experiments we have been unable to demonstrate that elution of the label is of any concern in our experiments

F Stohlman Jr If you vary the amounts of DFP in your incubation mixture while keeping the number of white cells constant is the rate of the loss of radioactivity altered?

A M Mauer We have varied in vitro doses of DFP^{32} from 0 1 to 1 0 γ/ml Within this range the curves have the same shape and $T\frac{1}{2}$ Amounts of *DFP* varying from 0 5 to 3 0 mg have been administered to normal subjects without influencing the disappearance curve

L E Osgood A crucial experiment might be done in mice You can get great numbers of leukocytes from the peritoneal cavity of mice These could be labeled in vivo and transfused by a technic similar to that of Gowans into isologous mice that have not had label Then the rate of accumulation in tissues could be determined or they could be injected intraperitoneally and the rate of appearance in the blood stream determined

E P Cronkite If one gives an amount of irradiation sufficient to eradicate new granulocyte production it takes at least five days to get rid of the granulocytes I don t see how this is compatible with Dr Mauer s observations

A M Mauer Following the destruction of the proliferating granulocytes in the marrow the number of granulocytes in the blood would not be expected to decrease until the existing store of maturing and mature granulocytes in the marrow had been exhausted Thus this type of experiment would measure the time required to deplete both blood and marrow stores

H M Patt Several years ago Dr Craddock[1] carried out studies with leukopheresis in x irradiated dogs and we used a leukocyte antiserum in similar experiments.[2] The experiments showed rather convincingly that there was a considerable store of relatively advanced neutrophil precursors This store was progressively depleted over several days after irradiation owing presumably to the block in new cell formation

I would like to comment on Dr Stohlman s reference to the incompatability between the thymidine data and the *DFP* results just presented I realize we are comparing dog and man However from the information that we presented using tritiated thymidine we suspect that in the beagle dog at least the turnover or disappearance time of granulocytes is of the order of 6 to 8 hours which is in the same range as the figure that Dr Mauer obtained with DFP^{32} in man

J M Yoffey We find that mature neutrophils stay in the marrow up to 12 days which bears out Dr Mauer s observations Why they don t leave I don t know

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Control of the Steady State Proliferation of Leukocytes

By CHARLES G CRADDOCK, JR , SEYMOUR PERRY
AND JOHN S LAWRENCE

THE DEMONSTRATION of humoral factor(s) regulating erythropoiesis, as described by Erslev and others may have implications extending far beyond the subject of erythrocyte production It is probable that similar lines of communication control the rate of proliferation of other growing tissues The signal for accelerated production is in response to an increased demand for cells this may result from loss or destruction of a portion of the tissue involved or from need for the particular tissue to increase its functional capacity It might have been anticipated that specific humoral factors affecting the rate of cell production would be first demonstrable for erythropoiesis because of the refined methods available for detecting alterations in red cell mass production rate and life span Quantitation of leukocyte kinetics is far more difficult It is obvious that the detection of specific factors controlling proliferation of leukocytes and platelets must await such quantitation

Changes in circulating leukocyte levels do not necessarily reflect alterations in production rate and, therefore, additional criteria are needed to assess production The myriad nonspecific factors that may influence blood leukocyte levels are too numerous to require discussion In order to ascribe a specific effect on leukopoiesis to a given agent the following must be shown (1) The agent must not exert any nonspecific effect such as inflammation or alterations in vascular dynamics That is it must not change the rate of removal, destruction or utilization of leukocytes in the periphery since loss of leukocytes in itself will stimulate production[4] (2) The effect of the agent must be an undisputed alteration in cell production The parameters of leukopoiesis are crude at present and consist chiefly of long sustained leukopenia or leukocytosis and morphologic alteration in the proliferative organ (marrow or lymphoid tissue) Acute fluctuations of peripheral blood leukocyte numbers even of considerable magnitude can occur without any change in the rate of proliferation[4] (3) The agent must exert its effect directly and not through some alteration in the entire body's hormonal balance The well established effect of adrenal steroids on lymphocyte proliferation, for example, can hardly be described as specific although the changes in lymphoid tissue after steroid administration may be more marked than in others

The purpose of this paper is to discuss some of the features of leukocyte kinetics that must be considered in the search for factors controlling leukocyte proliferation The mechanisms of control will only be briefly mentioned

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have migrated into extracellular spaces, or into the lumen of various hollow organs this remains to be proved That such may occur to some extent may not be denied, but one cannot assume that the blood granulocyte population exchanges equally with these extravascular cells

The endothelium of the capillaries of various organs represents a dividing line between intravascular and extravascular granulocytes, migration through the endothelial surface occurs continuously Viable granulocytes that are adherent to the endothelium may either go through or be released into the passing blood stream The ratio of the number adhering to the number moving through the capillary wall is unknown and varies with a host of factors principally the chemotactic forces outside the capillary and the rate of blood flow pressure and properties of the fluid medium within the capillary Assuming that the ratio is stable in the steady state, the number migrating out will bear a direct relationship within limits, to the number of cells within the capillary Ambrus and Ambrus have shown that a certain minimum number of granulocytes must be perfusing the lung before the organ removes cells from the perfusing blood [8] If the perfusate is cell free, the pulmonary capillary bed will release cell to maintain a certain minimal leukocyte level, but this supply of cells which can re-enter the perfusing blood is rapidly exhausted If, on the other hand, there is an excess of cells (i e , more than the minimal leukocyte level of about 1000 per cu mm) in the perfusing blood the lung will entrap cells until the perfusing level reaches this floor" The removal of excess cells may be continued to an apparently unlimited degree and is accompanied by the discharge of increasing numbers of cells into the bronchial secretions

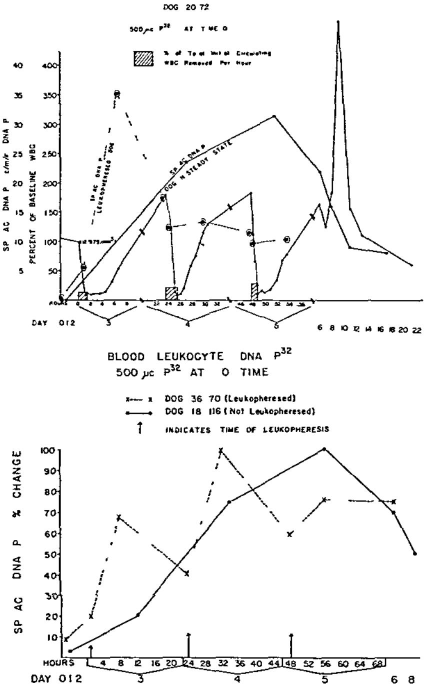
The granulocytes in the circulation must be considered compartmentalized in broad terms into those freely circulating in the larger vessels and those in capillary beds Interchange of cells occurs between these two compartments, but the net movement in the steady state is from the arterial blood into capillary blood and from the capillary blood into the tissues The length of time that granulocytes spend in capillary beds probably varies greatly in different tissues Therefore, there may be cells of widely divergent ages in the total circulation at any one moment

Additional evidence for this compartmentalization of the peripheral granulocytes is provided by leukopheresis In this experimental model which was designed to avoid as much as possible the introduction of factors that might alter capillary flow, the relationship between the cells in the central circulation and the capillary beds was quite evident When the number of circulating granulocytes was reduced to a minimum level (about 1000 per cu mm) a delay of 60 to 90 minutes was observed before the circulating granulocyte concentration began to rise after termination of leukopheresis This occurred in spite of the continued entry of cells from the bone marrow as shown by isotopic labeling of the marrow myeloid cells, and could be accounted for only by an accelerated distribution of cells From these data a conclusion similar to that of Ambrus and Ambrus was reached When maximum leukopenia is induced by leukopheresis recovery does not begin until a peripheral leukocyte pool has been primed with granulocytes This period of about 90 minutes is believed to be the time during which the capillary bed is repleted with a certain minimum number of cells following which the circulating cell concentration rises By estimation from the rate of rise in circulating cell numbers during the linear part of the curve the size of the capillary pool would be about three fourths that of the circulating pool before leukopheresis assuming that the rate of movement of cells from the capillaries into the tissues remains steady after the capillary bed has been repleted with granulocytes Thus it was calculated that cells were entering the blood at a rate which would replace those circulating every 2 hours the time required for filling the capillary bed is 90 minutes

2 *The Movement of Granulocytes Between the Blood and Tissues*

Data have been presented previously on the use of DNA labeling and have been interpreted as supporting the concept of a large intramedullary mass of granulocytes This mass has been estimated by Osgood[3] to be about 50 times the size of the circulating pool of granulocytes Patt[4] arrives at a figure of 26 times on the basis of his calculations Donohue and his associates[5] arrive at a ratio of 20 1 in man on the basis of Fe^{59} estimates of marrow cellularity The term marrow granulocyte reserve has been used to denote those cells which are in various stages of maturation beyond the point of DNA synthesis and subsequent division About one sixth of the marrow granulocyte population (myelocytes and their precursors) are believed capable of cell division In man there is a period of four to six days after the administration of the isotope before DNA labeled granulocytes appear in the blood In the dog this period is about three days This delay until the cells that were capable of a last division when the P^{32} was administered enter the circulating blood reflects the period in which these cells are held in the marrow granulocyte reserve At the time these highly labeled granulocytes are ready to move into the peripheral blood the entire marrow population of cells is labeled The unlabeled peripheral pool includes those cells freely circulating those in capillary beds and those out in the tissues body cavities etc Leukopheresis at the time when only the marrow population is labeled results in acceleration of the rate of entry of cells from the labeled marrow pool as shown by the sharp rise in granulocyte DNA P^{32} in the circulating cells This finding was interpreted by us to mean that the bulk of cells contributing to the leukocytosis after leukopheresis came from the marrow Several examples of this have been given[4] (figs 1 and 2) One experiment (fig 2) showed a fall in granulocyte DNA P^{32} over a period of some 20 hours after the initial peak of specific activity and then a subsequent rapid rise on the following day when leukopheresis was again instituted The experiment was interpreted by Osgood as follows Figure 2 conclusively shows dilution by unlabeled leukocytes from the tissue pool to give a lower specific activity at twenty four hours than at six or thirty two hours which proves the existence of the movement of cells (from extravascular areas back into the circulating blood) and is incompatible with the conclusion [3] The data do not permit differentiation of unlabeled cells re-entering from extravascular tissue spaces from unlabeled cells released from capillary beds as previously discussed

More explicit evidence that the movement of granulocytes is primarily one from the blood into the tissues was obtained by experiments in which a sterile peritoneal exudate was induced at various times in the course of appearance and



Figs 1 and 2 —The changes in blood granulocyte DNA P³² specific activity induced by leukopheresis first instituted on the third day after labeling, at a time when the marrow granulocyte mass is highly labeled in comparison to the peripheral granulocytes This is to be compared with the curve in a normal dog not subjected to leukopheresis

Leukopheresis causes an initial acute rise in specific activity which may be followed by a fall, as in figure 2 (*left*), due to dilution with unlabeled cells Whether these unlabeled cells re-enter from tissue spaces or are released from capillary beds cannot be determined from these data

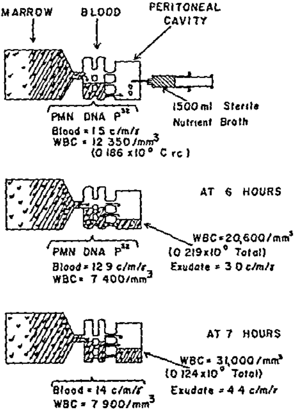


FIG 3 —The creation of a peritoneal exudate in a normal dog (No 1a 111) at a time when the marrow granulocytes are highly labeled (indicated by √) in comparison to the blood *The DNA specific activity of the granulocytes in the peritoneal exudate does not equal* that of the blood granulocytes, indicating a lack of free interchange of cells between peritoneal cavity and blood Note the fall in peripheral blood granulocyte numbers despite the rapid movement of cells into the exudate

disappearance of labeled granulocytes In this situation, cells migrate rapidly into the peritoneal cavity, but are not removed from the body If there exists an equilibrium between these cells and those in the peripheral blood, the specific activity of both cell populations will be equal Figure 3 shows the results in a *dog that received 1500 ml sterile nutrient broth intraperitoneally at 9 00 A M* on the third day after administering P^{32} (15 μc/kg) The peripheral blood leukocyte DNA P^{32} specific activity at this point was 1 5 counts/min/μg DNA P Six hours later the peripheral blood granulocyte specific activity was 12 9, the granulocytes in the exudate showed only 3 0 counts/min./μg The granulocytes numbered 20 600 per cu mm in the peritoneal exudate at this point At seven hours the peritoneal exudate specific activity was 4 4 counts/min/μg If the peritoneal exudate is induced at a time when the peripheral blood granulocytes are maximally labeled (fig 4), then granulocytes appear in the exudate in a matter of minutes These granulocytes have a specific activity similar to those in the blood If a major portion of the cells migrating into the peritoneal cavity were mobilized from a large extravascular mass of less highly labeled granulocytes, one would expect the specific activity to fall below that of the blood population These findings do not suggest a free exchange of cells

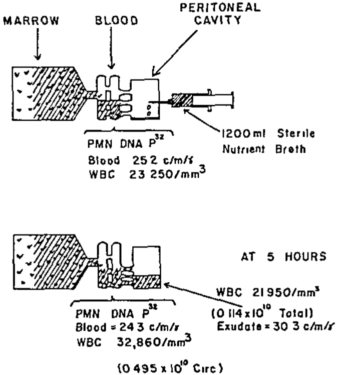


Fig 4 —A similar experiment to that shown in figure 3 with induction of peritoneal exudate at a time when the blood granulocytes are maximally labeled (Dog No 14 104) Here the specific activity of the exudate cells is as high or higher than in the circulating granulocytes suggesting that less highly labeled cells out in tissue spaces did not enter into the peritoneal exudate Note also that the removal of many cells from the blood does not cause a sharp fall in blood granulocyte specific activity

between the peritoneal cavity and the blood, instead they support the idea that the main movement of cells is from the blood into the peritoneal cavity

This type of data will be criticized on the grounds that such drastic manipulation of the animal vitiates any conclusion as to what may occur in the steady state This argument cannot be countered Indeed there is no means yet devised in which the movement of granulocytes can be approached without disturbing the steady state Nevertheless the bulk of evidence supports the contention that granulocytes perform most of their function in the tissues and that the majority of cells are utilized extravascularly The assumption that the cells circulating in the blood represent an average age of all the granulocytes in the periphery remains to be proved The importance of this assumption lies in the fact that many presently accepted estimates of granulocyte life span are based thereon

3 *The Relationship of Marrow Granulocyte Turnover to Blood Granulocyte DNA Labeling*

It has become evident that the rate of disappearance of DNA labeled granulocytes from the peripheral blood is not dependent upon the rate of removal or life span of these cells For example, individuals with known bacterial pyogenic infection (e g , lobar pneumonia) will show a fall-off of the curve which is similar to that of normal persons[14] Also, in the animal experiments previously cited, in which large numbers of granulocytes (several times the number freely circulating) were removed by leukopheresis or drawn into peritoneal exudates, there was not an associated disappearance of labeled cells from the blood

The significance of the blood granulocyte DNA curve becomes clear if the circulating granulocytes are envisioned as an extension of the marrow myeloid tissue In other words, the cells that enter the blood from the marrow are those cells which have completed their maturation and differentiation and, at a certain point in their development, are released into the blood The age of these cells entering the blood may be assumed to be roughly similar in the steady state (with certain exceptions to be discussed later) When the marrow myeloid cells are labeled during the period of DNA synthesis, and hence during proliferation, the labeled cells do not enter the blood until the completion of differentiation and maturation A sample of the peripheral blood granulocytes is, therefore, analogous to a sample of the most "mature layer" of cells in the marrow, i e , that portion of the marrow myeloid population which is releasing cells into the circulation There is general agreement that the delay of five to six days in the human before large numbers of labeled granulocytes enter the peripheral blood reflects the length of time before cells have progressed through this period of development It is our belief that the entire curve of granulocyte DNA labeling reflects the course of events in the marrow

Figure 5 is from a paper by Siminovitch and Graham[15] Here, a bacteria

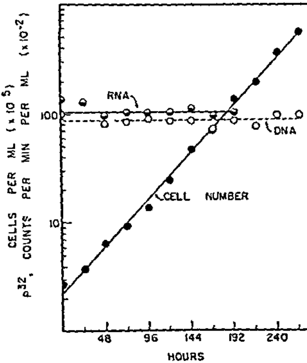


FIG 5—Retention of P^{32} in the RNA and DNA fractions of L strain mouse cells propagated in suspension by the rotating culture technic (from Siminovitch and Graham[15])

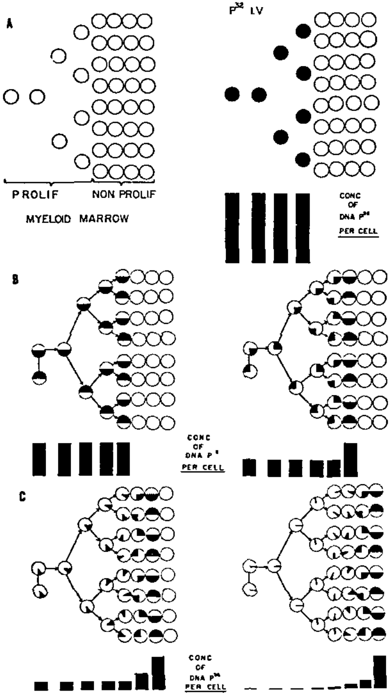


FIG 6 (A, B and C)—Schematic representation of one possible type of dilution of the DNA label by myeloid proliferation By the time the marrow reserve of nonproliferating granulocytes is labeled the most highly labeled cells enter the blood first See text for discussion

culture and a tissue culture have been labeled by P^{32} and then removed from additional P^{32}. The amount of DNA P^{32} remains constant for the entire culture during the period of logarithmic proliferation of cells. The concentration of DNA P^{32}, accordingly, is diluted at a rate that is the reciprocal of the increase in cell population. Of course, the situation in the intact animal is much more complex, and the problem of removal of the isotope or labeled precursors cannot be overcome. However, a similar phenomenon occurs and must be taken into account.

If those cells that give rise to granulocytes undergo several mitoses between the earliest stem cell and the final cell appearing in the blood, the curve of granulocyte DNA P^{32} reflects the specific activity of the progeny of all precursor cells capable of one or more divisions. Assuming, for the sake of clarity, that incorporation of the isotope occurs only over a brief period of time when the concentration of the isotope is high, that group of cells (e.g., the myelocytes) capable of only one subsequent division will give rise to the most highly labeled granulocytes. Those cells that will divide more than once after the labeling period will have their DNA P^{32} reduced by *one half* with each division. This is schematically represented in figure 6.

Those granulocytes that are the daughters of the last precursor cells capable of division, and hence the most highly labeled cells, move behind a large mass of more mature cells which are unlabeled and which precede the labeled cells into the blood. This process appears to be *one of an orderly progression according* to cell age, because highly labeled cells do not appear in a random fashion even when the rate of discharge of mature granulocytes from the marrow is accelerated, as by leukopheresis. It has been previously shown that repeated leukophere is *during the period when the marrow cell population is becoming labeled accelerates* the appearance of the labeled cells but does not alter significantly the *shape* of the specific activity curve. Leukopheresis at the time when these highly labeled cells are beginning to appear in the circulation will accelerate their delivery. However, fluctuations in the entry of cells into the peripheral blood *after* the marrow mass has become completely labeled (and the blood granulocyte specific activity has passed the peak) will change the slope of the curve very little. Indeed, the specific activity of the circulating granulocytes does not change its course *despite marked fluctuations in blood cell count (figs. 7 and 8)*.

These data reflect the fact that at the time the first cells which are labeled have moved through the marrow mass of more mature cells and are ready to appear in the blood, the entire marrow myeloid mass of cells is labeled, and the extent of labeling of each group of cells from oldest to youngest is progressively diluted. Ottesen has attempted correction of the blood granulocyte DNA curve for the amount of P^{32} available for continuous incorporation into DNA. The resulting curve is that which might be expected from the type of DNA P^{32} dilution discussed above (fig. 9).

Ottesen's calculations provided him with a *mean age* of the circulating granulocytes of about nine days. It is to be noted that Ottesen did not draw any conclusion as to the total life span of the cell. A mean age of nine days *does not* tell us whether there is an equal distribution of cells younger and older than nine days, nor does it tell us whether the cells enter the blood before the ninth day

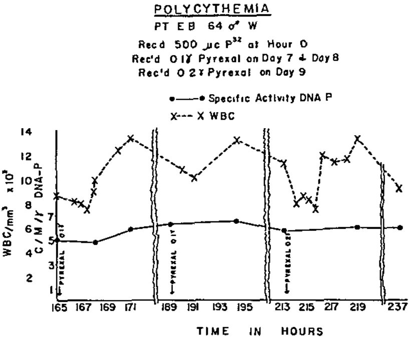


Fig 7—Failure of fluctuations of blood granulocyte numbers brought about by intravenous pyrogen to alter the blood granulocyte specific activity in a case of polycythemia vera

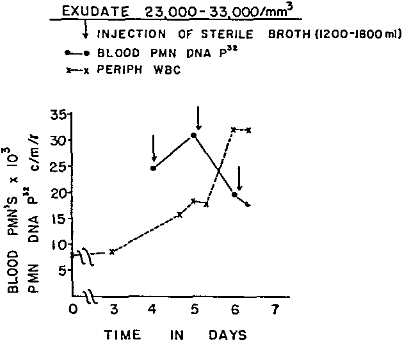


Fig 8—The repeated intraperitoneal injection of sterile broth, mobilizing large numbers of granulocytes from the blood and marrow, fails to cause the disappearance of labeled cells from the blood

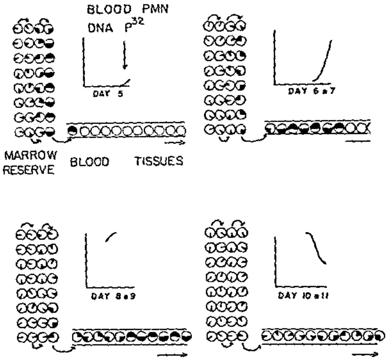


FIG. 9.—Schematic representation of the type of DNA specific activity curve that would be expected in the blood granulocytes as a result of the dilution of the DNA label by proliferation and by release of cells from a labeled marrow mass of nonproliferating cells.

One cannot apply the Volterra formula to this type of curve to calculate granulocyte life span. The situation is unlike that of N^{15} glycine labeling of erythrocyte hemoglobin in several respects.

The general shape of the blood granulocyte specific activity curve is affected very little by acute changes in the circulating mass of cells. In fact, the only portion of the curve that can be readily changed is the rate of rise in specific activity, once highly labeled cells have begun to enter the blood. After the entire marrow mass of cells has become labeled, the volume of labeled marrow cells is so much larger than that of the blood that acute fluctuations in the blood level affect the total marrow labeling very little. This relationship suggests a very rapid "turnover" of cells in the peripheral blood under normal circumstances. Even acceleration of the peripheral blood granulocyte "turnover," by infection, does not deplete the marrow of cells to a degree where the fall-off of the curve is altered.

Another factor offsetting a more rapid fall off of the curve in infection or in other situations where granulocytes are being utilized more rapidly than normal, is myeloid hyperplasia at the time of DNA labeling. If there is accelerated mitotic activity, the number of cells capable of DNA synthesis is increased, therefore, the number of highly labeled granulocytes entering the marrow reserve is increased, and the more intense labeling of the marrow myeloid population tends to offset the more rapid disappearance of labeled cells in the tissues.

If the marrow mass of nonproliferating myeloid cells is estimated, and the time of turnover of this population is known, it is a simple matter to calculate the production rate of granulocytes Osgood[3] has estimated the marrow myeloid tissue at 2 4 × 10^{10} cells per Kg , or in the 70 Kg man, 168 × 10^{10} cells, of which one sixth are in the proliferating group this gives a marrow myeloid reserve of 140 × 10^{10} granulocytes If this reserve requires six days to turnover, 23 3 × 10^{10} cells per day, or roughly 1 × 10^{10} per hour, are entering the blood If O good s estimate of circulating cells numbering 2 8 × 10^{8} per Kg (or 2 0 × 10^{10} in the 70 Kg man) is used, this means that enough cells are entering from the marrow to completely replace those circulating every two hours

Patt's estimate[12] of the marrow mass of nonproliferating granulocytes is considerably lower than Osgood's 512 5 × 10^{9} in the 70 Kg man If his value for the blood granulocyte pool, 25 × 10^{9}, is used the rate of entry of cells from the marrow is 85 4 × 10^{9} per day, or about 4 × 10^{9} per hour The circulating mass would be replaced every six hours Patt calculates, on the basis of comparing the mitotic activity of granulocytic tissue with the known production rate of erythroid tissue, that the production rate of granulocytes is 8 2 × 10^{9} per hour, approximately double that estimated on the basis of the turnover ' time of his calculations of marrow granulocyte reserve This figure is actually in fair agreement with the estimate (10 × 10^{9} per hour) derived from O good's calculation of marrow cellularity It is conceded that all such attempts are rough approximations and that a small difference in the estimated mitotic index, intermitotic interval, etc will give results that differ widely The point of general agreement, however is that sufficient numbers of cells are produced in the marrow to replace those circulating in a few hours Even if the number of granulocytes in the peripheral blood in the steady state is doubled to include those in capillary beds as well as those freely circulating the average time within the vascular system is a matter of 4 to 12 hours

The same factors that complicate the interpretation of DNA specific activity curves apply to any procedure that labels the marrow population of cells as well as the circulating cells Thus the studies with Cr^{51}, S^{35} 1 cystine and DFP^{32} reflect the movement of labeled cells from the marrow into the blood and the dilution of the label by cell division It is of interest that the granulocyte life span is calculated by most of these methods is close to 13 days Our interpretation of these results would be that the time required for the marrow population of cells to replace itself is about 13 days

The kinetics of granulocyte proliferation described above do not hold in conditions associated with disorders in the growth of myeloid tissue In active leukemic states, whether chronic or acute myelogenous leukemia, the proliferating mass of cells is no longer confined to the marrow Granulocytes are released into the blood from the marrow or from extramedullary areas of myelopoiesis before they have progressed through the period of maturation and development which normally occurs in the marrow myeloid reserve " This is reflected by an alteration in the rate of appearance of significant DNA labeling in circulating granulocytes[14] Indeed the rapid increment in circulating leukocyte DNA P^{32} in certain instances of acute leukemia reflects the active DNA synthesis in circulating cells, which can be shown by in vitro labeling of such leukemic cells For this reason, the data derived from such studies of leukemic patients are not comparable to the study of the steady state situation It is of interest in this

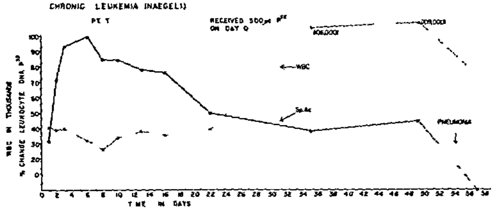


FIG 10—The rapid appearance, but slow disappearance, of leukemic cells whose DNA has incorporated P^{32} in a case of chronic leukemia Naegeli type

regard that our results have not shown a more rapid disappearance of DNA labeled cells in various myeloproliferative disorders Figure 10 shows the early appearance of labeled cells in a case of monocytic, myelogenous leukemia The continued presence of labeled cells is noteworthy, as is their disappearance during a fatal infection In the context of the foregoing interpretation of the DNA P^{32} curve reflecting the turnover of the entire proliferating mass rather than the cell life span, the very prolonged fall in the curve would mean that the mass of proliferating tissue is very large, in relation to the mass of cells in the circulation Hence, the time required for this proliferating tissue to dilute the DNA label by cell division is prolonged That leukemic tissue may in some *instances require a longer time for mitosis than normal has been shown by* Lajtha[9] and others

The Lymphocyte

There is considerable confusion about the kinetics of lymphocyte production and distribution, as has been amply described by Yoffey,[3] Ottesen[11] Hamilton,[3] Osgood,[3] Gowans,[7] and others DNA labeling experiments have indicated a very prolonged life span for the bulk of lymphocytes, although some of the data have been interpreted as showing a small percentage of cells with a short life span The chief characteristics of the blood lymphocyte DNA labeling curve are rapid appearance of labeled cells, but a very low maximum specific activity when compared with the granulocyte, and the presence of cells labeled to this degree for a long period of time

These findings, which have provided an estimate of lymphocyte life span of 100 to 300 days, must be reconciled with the evidence indicating rapid proliferation of lymphoid tissue This paradox has been explained by various means, such as the estimation of a very large extravascular and extrahematopoietic mass of lymphocytes that has a very prolonged turnover ' Furthermore, it is suggested that the lymphocyte contributes large fragments of its DNA to other presumably immature lymphocytes, and hence the isotope data provide an artificial prolongation of cell survival Others have suggested that the lymphocyte differentiates into a variety of other cell species, as stated many years ago by Maxi mow, Jordon, and others. It is not clear how such an occurrence would provide the long lymphocyte life span indicated by the DNA labeling data.

A great deal of attention is being devoted to the lymphocyte's apparent ability to govern the genetic transmission of antigenic specificity (in the homograft reaction), as well as to its role in antibody globulin synthesis. Recently the hypothesis has been presented ascribing to lymphocytes the role of supplying proteins and nucleic acids to practically every proliferating tissue in the body[8] As Dr John S Lawrence remarked recently, the lymphocyte would seem to be the most potent cell in the body, except for the spermatozoa In any event, it is probable that the fundamental activities of the lymphocyte complicate precise definition of kinetics

The difficulties of interpretation of DNA labeling in terms of cell kinetics, as described for the granulocyte, also apply to the lymphocyte In fact, the complexities become even greater when the possibilities of recirculation between blood and lymph are added For these reasons no reliable definition of the rate of production, distribution, life span, etc, is now possible for the lymphocyte Several observations are pertinent to clarification of existing misconceptions and for future elucidation

From the study of dog thoracic duct lymph it is clear that two cell types exist About 15 to 20 per cent of the cells are large with reticular chromatin and nucleoli, the remainder are typical small lymphocytes with dense nuclei and scanty cytoplasm The large cells show the same type of motility as the small ones, but are much more active Mitosis occurs in the large cells, but has never been observed in the small cells This is similar to the observations of Gowans on rat lymph, in which species the large lymphocytes comprise less than 5 per cent of the cells In dog lymph these large cells actively incorporate P^{32} into DNA upon incubation with the isotope in lymph They also avidly incorporate tritiated thymidine The small cells have never been observed to do either Human lymphocytes obtained from a patient with chylothorax show rapid DNA synthesis, although the percentage of cells capable of DNA synthesis is unknown

If the dog's thoracic duct lymph is followed after parenteral P^{32}, a characteristic specific activity curve is obtained There is a rapid rise to a maximum at about eight hours This maximum is very much higher than that observed in the blood lymphocytes All of the DNA P^{32} is contained in the large lymphocytes There follows a slow decline to a prolonged plateau of low specific activity, which is at the same level as for the blood lymphocytes The pattern of this curve is independent of the number of cells passing through the lymph[14] Although direct evidence is lacking, it would appear that the prolonged low specific activity of both blood and lymph is due to labeling of the small lymphocyte population because the specific activity is the same in both areas, although the percentage of large lymphocytes is higher in the lymph It has not been established whether the circulating small lymphocytes are products of the larger cells Our observations with phase microscopy of dividing cells agree with those of Gowans[7] in the rat, which showed that the products of division of these large cells retain the morphologic appearance of the large cells and not of the small lymphocyte

The very high level of DNA labeling observed in the large lymphocytes in thoracic duct lymph is not detectable in the blood lymphocytes (fig 11) This is

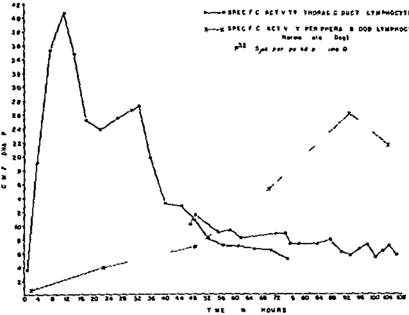


FIG 11 —Thoracic duct lymphocyte DNA specific activity in the normal dog compared with the peripheral blood lymphocyte specific activity in a normal intact dog receiving similar amount of P^{32}

due to the dilution of these cells with unlabeled small lymphocytes The large highly labeled cells seem to disappear rapidly from the blood and presumably are removed in some tissues possibly in the bone marrow as suggested by Yoffey[3] and Fichtelius[6] or in lymphoid structure spleen etc Studies in this laboratory with dog lymphocytes tagged with chromium phosphate (P^{32}) indicate that lymphocytes (both large and small lymphocytes are tagged by this method) do enter the bone marrow and remain there for several days. However we are unable to say whether the bone marrow removal is greater than that which occurs, for example in the liver and spleen Studies are under way in which the large lymphocytes are labeled specifically with tritiated thymidine and then re infused it is hoped that the fascinating problem of the fate of these cells can be clarified by this technic

It is obvious that the blood lymphocyte DNA curve is highly complex The early appearance of labeled cells would be expected because there is no delay before their entrance into the blood, as in the case of the granulocyte that enters behind a mass of unlabeled cells Despite high specific activity of a few lymphocytes at least initially the total blood lymphocyte specific activity remains low because of dilution of the sample with unlabeled cells It seems evident therefore, that from the standpoint of tissue growth when we speak of the lymphocyte, we must qualify the term and not consider all lymphocytes as cells with similar capabilities It may be that the large lymphocytes are more important as precursors for other cells and that the small variety has more to do with protein synthesis For example Gowans has presented very effective experiments[7] that support the suggestion of Mann and Higgins[10] that sustained lymphocyte production is prevented if a small percentage of viable proliferating cells do not reach the blood from the lymph We are all familiar with the very rapid lym phopenia that develops after irradiation. It was shown many years ago that x ray caused a rapid decrease in the output of thoracic duct lymphocytes in cats. Adrenal cortical steroids exert a very profound effect upon lymphoid tissue, and their suppressive action on cell proliferation in a variety of tissues is well known. All of these data indicate that lymphocytes disappear rather quickly if production is terminated. This is at variance with the concept of a very long life span for these cells and suggests that the estimates of lymphocyte survival time by DNA-labeling methods are artificial, as discussed by others.

These findings also suggest that lymphocytopoiesis is not confined to organized lymphoid structures but occurs in the flowing lymph, the blood, and probably in a variety of other tissues. These cells are, in a sense, comparable to metastatic or leukemic cells with a potential of transplantation to a variety of tissues in which they proliferate. Studies of the homograft reaction support the concept of certain lymphoid cells being capable of extralymphatic proliferation. For example, Terasaki, Longmire and Cannon[16] have shown that the injection of viable adult lymphocytes into chicken embryos results in a "graft against host" reaction, leading to the death of the chick with splenomegaly and anemia. The same phenomenon has been shown in embryonic mice injected with adult mouse lymphocytes, obtained from the thoracic duct. The reaction is believed to result from proliferation of the transplanted cells which eventually subdue the host because of immunologic incompatibility. In this instance, the embryonic recipient is tolerant of the adult cells, but the latter, as the host matures, react against the host cells as "foreign."

Because cells of this type are constantly entering the blood via the lymphatics, and because they appear to proliferate in various body tissues, it is conceivable that the rate of proliferation and, hence, lymphocyte production may differ from one area to another, depending upon vascularity and other environmental factors. It is suggested that the prolonged presence of DNA labeled lymphocytes may reflect, in part, the entry of lymphocytes into the blood from different sources and at different rates.

We have noted marked fluctuations in the lymphocyte DNA specific activity curve in patients with chronic lymphocytic leukemia.[13] These multiple irregular peaks suggest the erratic appearance of labeled cells from different areas and at different rates. Furthermore, as was pointed out in the discussion of granulocytopoiesis, the kinetics of the cell DNA curve reflect events in the proliferating tissue. In lymphocytopoiesis, the proliferating tissue is not confined to a specific organ. Ablation of proliferation of these widespread cells, as by whole body irradiation or adrenal steroids, leads to a rapidly developing lymphocytopenia, since production in all these diverse areas is terminated.

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16 Personal communication

Discussion of Regulation of Granulocyte Production

A S GORDON I would like to present some of our recent experimental evidence which might be construed as supporting the concept of a leukopoietic stimulating factor, the existence of which was inferred by Drs Craddock and Dougherty This work was done in collaboration with Dr M Eisler and the Messrs R O Neri, B S Dornfest, C D Siegel, E Handler and A Crusco Influenced by the now well established finding that erythropoietin can be evoked in the circulating blood by removal or destruction of erythrocytes, it seemed possible that removal of large numbers of leukocytes might result in the appearance of a leukocytosis-inducing factor

Parabiotic rats were employed in the first series of experiments Leukocytes were removed from one of the partners of each pair by a method of peritoneal cavity lavage Twenty five ml of nonpyrogenic sterile isotonic saline was injected intraperitoneally into each of the right parabionts Nine hours later, an additional 10 ml of saline was injected to permit collection of the leukocytes which had accumulated in the peritoneal cavity This procedure, repeated six to eight times, enabled us to remove approximately a billion leukocytes over a two to three day period Determinations made initially and after termination of the final leukocytapheresis (LAP) showed that the peripheral white cell counts (WBC) had decreased or remained un changed in the right LAP partners of the three pairs of parabionts reported here (Discussion table 1) On the other hand an average increase of 65 per cent had occurred in the WBC of the left non LAP members These increases were the result of elevations in the numbers of both polymorphonuclear (PMN) and lymphocytic elements

In the second series of experiments, rats were LAP on six to eight occasions via the peritoneal cavity until more than a billion leukocytes had been removed Single subcutaneous injections of 10 ml of plasma, obtained from these rats, into normal intact rats of the same strain resulted in a mean increase of 130 per cent in the circulating WBC at four hours (Discussion table 2) Both PMN leukocytes and lymphocytes contributed to this increase Injections of 10 ml of plasma from normal non LAP rats into six normal rats resulted in no significant alterations in the WBC of the recipients (Discussion table 3) Injections of adrenal cortical steroids, serotonin adrenaline nor epinephrine histamine and Piromen did not reproduce the pattern of leukocyte response evoked by the plasma from LAP rats In addition, conventional methods for the detection of microorganisms failed to reveal their presence in the plasma of LAP rats

DISCUSSION TABLE 1—*Parabiosis experiments Effects of repeated leukocytaphereses (LAP) in right parabionts on the leukocyte counts of both partners*

Parabiotic pair number	Right partner (LAP)		Left partner (non LAP)	
	B*	A*	B*	A*
1	14 6	8 8	12 6	20 0
2	16 9	9 1	15 0	31 0
3	15 0	15 3	17 0	25 0

* B Leukocyte counts (thousands/mm^3) before LAP

* A Leukocyte counts (thousands/mm^3) after LAP

DISCUSSION TABLE 2—*Effects of a single injection of 10 ml plasma from LAP rats on the peripheral leukocyte counts of intact rats*

Recipient rat number	Leukocyte counts (thousands/mm^3)	
	Initial count	4 hrs. after inject
1	12 8	30 0
2	23 0	45 0
3	17 0	37 5
4	8 4	20 7
5	7 6	18 0
6	11 2	29 0

The third series of experiments was concerned with testing the effects of plasma from LAP rats upon the rate of release of leukocytes from isolated hind legs of rats perfused with approximately 50 ml of leukocyte-free blood Approximately 5 × 10^7 leukocytes were released into the per

DISCUSSION TABLE 3—*Effects of a single injection of 10 ml plasma from normal (non LAP) rats on the peripheral leukocyte counts of intact rats*

Recipient rat number	Leukocyte counts (thousands/mm^3)	
	Initial count	4 hrs. after inject
1	19 9	21 1
2	15 0	15 9
3	16 9	17 5
4	15 0	16 8
5	17 5	18 5
6	16 5	17 0

DISCUSSION TABLE 4—*Effects of plasma from LAP rats upon the mean numbers (millions ± standard errors) of leukocytes released from perfused hind legs of rats over a 30 minute period*

Group number	Total leukocytes released	Granulocytes	Lymphocytes
1 (5 expts) controls (no LAP plasma added)	53 2 ± 6 8	34 8 ±3 4	18 4 ±4 5
2 *(3 expts) 7* ml LAP *added*	*131 9* ±11 0	*60 0* ±4 0	*72 0* ±6 0
P values	<0 01	<0 01	<0 01

fusate from such hind legs in a 30 minute perfusion (Discussion table 4) Addition of 7 ml of plasma from repeatedly LAP rats at the initiation of the perfusion resulted in the release of approximately 150 per cent more leukocytes into the perfusate over the same time interval This increase achieved with the LAP plasma was the result of the release of larger numbers of both PMN and lymphocytic elements Addition of the same amount of plasma from normal non LAP rats failed to increase the rate of leukocyte release from these perfused preparations

These three lines of evidence lend support to the thesis that circulating leukocytosis-promoting factors exist which are concerned with replacement of leukocytes removed from or destroyed within the body Experiments are under way to determine whether this action is one exerted not only on leukocyte release but upon leukocyte production as well

A M MAUER Although we have presented evidence that granulocytes are removed from blood in a random fashion we cannot tell from our data whether they enter the blood from the bone marrow in a random or sequential fashion Both types of model have been analyzed in the analog computor but unfortunately both models can be made to fit the data curve

After the injection of P^{32} in the form of sodium phosphate as described by Dr Craddock there are several factors which influence the rate of increase of radioactivity in the DNA of blood leukocytes The period of availability for incorporation into DNA would greatly influence the radioactivity of cells discharged from this pool Once the labeled cells left the proliferating pool a random distribution about a mean time spent in the maturation pool might be expected and this also would influence the rate of increase of labeled cells in the blood Finally if granulocytes are removed from the blood in a random fashion the rate of increase of label in the granulocyte DNA would be influenced by the rate of removal of cells from the blood With these possible factors influencing the rate that P^{32} appears in the DNA of blood granulocytes measurement of the rate that granulocytes are delivered to the blood by this method may be only an approximation

C G CRADDOCK Dr Gordon has touched on something that has bothered those of us who have been concerned with separating release of cells from proliferation of cells I think that he is correct in being conservative in interpreting his data There are many nonspecific factors that can affect leukocyte removal in the periphery thus triggering an accelerated release of leukocytes without any change in the rate of proliferation As was pointed out by Dr Patt if one irradiates an animal and stops all proliferation one can still cause the same type of release of cells from the intramedullary reserves

I am not sure that we are in conflict with Dr Mauer In the studies on P^{32} labeled DNA cells begin to appear on the fifth to sixth day, the linear portion of this curve occurs over the fifth to seventh day then there is a slower rise to a maximum on the ninth day The dilution by the entry of less highly labeled cells of course occurs earlier so that the maximum point on the curve does not represent the point of highest labeling per cell in the peripheral blood This point occurs earlier I feel that we are in agreement as far as the rapid turnover of cells in the peripheral blood is concerned

L G LAJTHA Another slightly complicating factor in connection with what Dr Craddock mentioned is that when we look at the DNA labeling of different bone marrow cell populations there are many different compartments If we take the earliest blast cells the so-called pro cells and the late meta type cell then the grain counts over these three types during any time will usually vary in the relation of 1 2 0 5 Hence we can t treat the labeled bone marrow population as uniformly labeled

A M MAUER If granulocytes are removed from the blood in a random fashion highly labeled cells have the same chance of removal as unlabeled cells The blood compartment in your model is represented as a tube cells being removed in a sequence determined by the time of entry Our data do not support such a removal mechanism

C G CRADDOCK Had I known about your model T before I drew my diagram I wouldn t have drawn a tube I should like to emphasize that we do not feel that there is an orderly movement of cells through the peripheral blood according to age As a matter of fact all of our data are to the contrary We feel that the turnover of the blood pool is so inconsequential in terms of mass in relation to the marrow reserve of cells that its fluctuations do not affect our curve significantly

A M MAUER It is very gratifying that we are in agreement as to the type of removal of granulocytes from blood

V P BOND With tritiated thymidine the percentage of labeled cells in the peripheral blood is determined by a variety of factors principal among which are the percentage of labeled cells in the dividing precursor pool the rate at which labeled cells are removed from the peripheral blood and the rate at which labeled cells are apparently lost through dilution of grain count It appears that the time for the maximum percentage of labeled cells in the peripheral blood would probably represent the resultant of the first two of these factors Any sharp change in the percentage of labeled peripheral cells represents principally a change in the percentage of labeled dividing precursors and the time for such a radical change to occur probably represents an upper limit for the generation time of the dividing precursor cell

E P CRONKITE I find the idea of random loss perfectly acceptable and I think this would be an appropriate way for the granulocytes to be taken out of the circulation for some purposeful function However when you start to put in a $T^{1/2}$ of a few hours I begin to stumble and I just want to review very quickly the things that Dr Bond presented About 36 hours after injection, labeled cells begin to appear in the peripheral blood Between the sixth and seventh day you see the maximum percentage of labeled cells During the time that the percentage of labeled cells increases, the grain count per cell is essentially the same To me this is the minimum time from the last myelocyte division to emergence You go from zero to the maximum percentage of labeling which is essentially the percentage labeling seen in the marrow This takes three days roughly There is approximately a 40 per cent increase per day To increase the percentage *of cells labeled a labeled cell must replace* an unlabeled cell since some type of steady state exists However the rate of rise in percentage labeled cell does not appear to me to be compatible with a peripheral blood half time of six hours nor a marrow storage of nine to eleven days

D L KLINE We have attempted to demonstrate in the dog and in the rabbit whether or not extravascular lymphocytes might return after depression of the lymphocytes by hydrocortisone All of our evidence so far indicates that the cells which make up the blood level after the low point following hydrocortisone has been reached have come from the lymph nodes

M A SCHNEIDERMAN Several speakers talked about problems of life span but I couldn t tell what really was meant by life span I would like to put this in terms of a simple analogy that perhaps one can follow more easily What is the life span of a man? Is the life span of a man the maximum life which he can attain? I think this is what Dr Odell refers to Is it the average life expectancy at birth? Is it the average age of the population that died today? To carry the analogy further shall we take gestation period as part of our estimate of life span? If we take gestation period for the measurement of life span how shall we consider fetal wastage through miscarriages and so on? This is Dr Stohlman s point, the death of the cells in the early operation being equivalent to fetal wastage If we take fetal wastage should we include the wastage of germ cells that don t eventuate in fertilized germ cell ?

Frankly I don t know what we ought to do I can at the time of birth put an orange stamp on a man s forehead and I have now labeled him Every time someone is born I do likewise I watch the population until finally I get to the point where everybody has a orange stamp on the forehead I record how long this process takes Have I now measured the life span?

I don t know which one of these many possibilities one means when they say life span but whatever is meant should be defined

L M TOCANTINS That is very fine Just those thoughts were going through my mind

C G CRADDOCK I think to quote a *famous cigarette slogan you should let* each man make his own decision I don t see what s so terrible about starting the measurement of life span with the gesta tional period when we can measure the gestational period Apparently, we can t measure adult ' life of the granulocyte and this is the nub of the problem As has been repeatedly said, no one can say how long a given granulocyte will live once it leaves the circulation I think it depends on where it happens to leave If it leaves in the middle of the stomach, it undoubtedly will, live a very short time If it leaves and enters a tissue space with a comfortable environment it may live for quite a while

Adrenal Cortical Control of Lymphatic Tissue Mass

By THOMAS F DOUGHERTY

IN 1943, DOUGHERTY AND WHITE[12] discovered that single administrations of ACTH to several species of animals was followed by a rapid loss of lymphocytes from the blood The loss became maximal in three to nine hours, depending on the preparation, the amount of ACTH administered, and the particular species studied This rapid loss was followed by a rapid reconstitution to normal lymphocyte levels, which, in all instances were back to the normal homeostatic level within a period of 24 hours This phenomenon—a rapidly developing lymphopenia, followed by a rapid reconstitution to normal numbers of blood lymphocytes—was called the lymphopenic response

The specificity of adrenal cortical hormones for the production of the lymphopenic response was demonstrated by the fact that ACTH could not produce it in the absence of the adrenal cortex[13] It was further found that those substances now called cortisol (compound F Kendall) and cortisone (compound E-Kendall) were the particular adrenal cortical hormones that produced the lymphopenic response[13] Measurements of the relationship between blood level of 17OH corticosteroids and the time of induction of lymphopenia were made by Nelson et al[22] They found that cortisone acetate and cortisol acetate produced increased levels of blood corticoids one hour after intramuscular administration although maximum lymphocytic changes did not occur until four to eight hours At this time the blood hormone level was either normal or below Among steroid hormones only adrenal cortical steroids produce the lymphopenic response in adrenalectomized animals[13] Many other hormones such as insulin epinephrine and thyroxin, as well as certain steroid hormones produce a lymphopenic response when they are administered to intact animals but not when they are given to adrenalectomized animals[16]

METABOLISM OF CORTISOL

Cortisol, which is the major lymphopenia producing steroid in human is secreted from the adrenal cortex into the blood It rapidly leaves the blood and enters the loose connective tissue that embeds the capillary[8] The hormone is taken up here by local tissue elements primarily fibroblasts[7] which metabolize it very rapidly, thus converting cortisol to other steroid molecules[2] not having a lymphopenic effect These products and cortisol itself, pass back from the tissue into the blood and circulate through the liver at which point they are further catabolized, but in a different fashion[8] The liver is the major organ capable of reducing the A ring, reducing the 3 keto position and forming water soluble conjugated steroids (i e , glucuronides), which are then excreted[3] The action of the liver is absolutely essential to create molecules that can then be excreted

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but extrahepatic cells also convert cortisol to inactive lymphocytolytic steroids The action of the liver, then, is to provide the drain on this cyclic system

It is important to realize that these actions of both the cells of connective tissue and liver are exceedingly rapid For example, in the human being, conversion products from cortisol to nonlymphopenia producing hormones are found within five minutes in the urine of individuals given C^{14} labeled cortisol[1] The half life of free cortisol—not of its conversion products, but of cortisol itself—in the blood of man and mouse is about 40 to 50 minutes, and by 100 minutes virtually all the administered cortisol is removed[8] Thus, cortisol is available to act upon the lymphatic tissue for a very short time following any one increment in the amount in blood It is evident that however cortisol affects lymphatic tissue, this effect is maintained for a longer period of time than free cortisol is actually available to act upon the cell[8] In order to produce a lymphopenia, the amount of free hormone in the blood must be greater than that characteristic for the homeostatic amount This condition can be produced when the rates of synthesis and secretion of cortisol are greater than its rates of inactivation and excretion Conditions such as a continuous supply of ACTH in a quantity sufficient to maintain a prolonged hypersecretion of cortisol (Cushing's syndrome) or situations of prolonged stress, result in lymphopenia, the duration of which depends on the length of time that the cortisol level is above normal[24] On the other hand, in the presence of interference with free hormone conversion or degradation, as found in liver injury, more free cortisol is available Patients with cirrhosis of the liver have very high levels of free cortisol and marked lymphopenias[17] Other unknown factors, of course, could enter into this picture by interference with hormone degradation

The statistically normal number of blood lymphocytes is maintained within limits when a statistically normal amount of free cortisol is present in the blood (0 8 μg /10 cc human plasma) When the blood level is greater, the lymphocyte count decreases Conversely, when there is an inadequate supply of hormone, there is a tendency for a lymphocytosis (fig 1)

MEDIATED LYMPHOPENIC RESPONSE

The hormone supply (cortisone, cortisol) necessary to maintain the homeostatic lymphocyte level (effective lymphocyte maintenance dose) was found to be 50 μg /20 Gm mouse/day for cortisone, and 10 to 15 μg /20 Gm mouse/day for cortisol[20] The daily maintenance dose would be in the neighborhood of 35 mg / day of cortisol for a 70 Kg man This is within the approximate range of cortisol required to maintain adrenalectomized individuals in a eucorticoid state These approximate doses are of importance when it is considered that the adrenal cortex influences markedly the rate of growth and development of lymphatic cells, and that the lymphocyte count reflects this general influence The number of blood lymphocytes, therefore, gives an accurate indication of the amount of circulating free cortisol

The effect of stress on the production of a lymphopenia is mediated by way of the pituitary adrenal axis The lymphopenic response may accompany adaptive physiologic alterations such as pregnancy, lactation, emotional stimuli, and

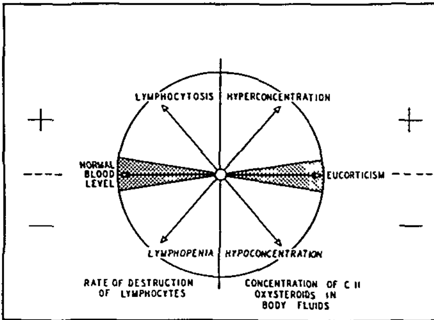


FIG 1—The relationship between the concentration of lymphocytolytic hormones in blood and the number of lymphocytes

a wide variety of situations in which there is endocrine imbalance [6, 16] Starvation, which is one of the most potent means of depleting lymphatic organs, is ineffective in the adrenalectomized animal [18] The fact that a wide variety of traumatic, bacterial, and chemical agents produce lymphopenia by this mediated response is recorded in many reviews on the subject Indeed, there are only two lymphopenia producing agents that are effective in the absence of the adrenal, namely, x irradiation and nitrogen mustards Even a portion of the effect of these agents is mediated by their induction of adrenal cortical secretion [15, 16]

The lymphopenic response that follows stress differs from that produced by *lymphocytolytic hormones, in that there is an increase in blood lymphocytes for* a short time before the lymphopenic phase begins [6, 16, 21] The relationships of stress to changes in blood lymphocytes may be explained by defining five phases of a curve of response of circulating lymphocytes to stress[20] (fig 2) During phase 1, there is an increase in the number of lymphocytes immediately following stress in both intact and adrenalectomized animals [17, 21] Phase 2 is the rapidly developing decrease in circulating lymphocytes, which is mediated by adrenal cortical secretions in intact animals Phase 3 is the curve of the rate of reconstitution of *circulating lymphocytes from the maximal lymphopenic level to the normal* limits Phase 4 is a lymphocytotic overshoot, which is frequently present following more severe stressful circumstances That is, when the rate of reconstitution is great, the lymphocytes will increase over the physiologic norm this is followed by phase 5, which represents the reconstitution from phase 4 to the statistically normal circulating lymphocytes characteristic of prestress levels

Phase 1 has been attributed to epinephrine secretion,[21] which theoretically produces splenic contraction with a subsequent discharge of cells This explana promoting substances Thus more hormone than the statistically normal amount is required to dampen the phase 4 overshoot and bring the cell number into the range characteristic for homeostasis There may be a considerable amount of oscillation about this mean level before a normal level is reestablished

Lymphocytotic Response

During our studies of the involution of lymphatic tissue and the lymphopenia induced by stress it became evident that the blood lymphocytes tend to increase in number following adrenalectomy to higher mean levels with smaller standard deviations than in intact animals There is no progressive increase of lymphocytes in adrenalectomized animals i e they increase significantly but remain at this new level until death It was found however that when stressful agents were administered to the adrenalectomized animals increases far greater than those due to adrenalectomy alone occurred[11] For example two hours following adrenalectomy various stressors that produced a lymphopenic response in intact animals were given or applied to the operated mice Almost immediately the blood lymphocytes rose to levels significantly higher than those present in the nonstressed intact or adrenalectomized group[11] Particular attention was paid to three types of stressors histamine administration endogenous histamine and *serotonin release by way of anaphylactic shock and starvation*[20] *Other stressful* agents such as insulin pitressin adrenalin thyroxin various androgenic and estrogenic hormones have all been investigated and reported[6] All of these agents produce a lymphocytosis in the absence of the adrenal gland

The fact that blood lymphocytes do not increase progressively but remain at the level found 24 hours after adrenalectomy suggests that there are mechanisms other than those mediated by secretions of the adrenal which regulate the proliferation and destruction of blood lymphocytes When the dampening effect of adrenal cortex is absent it is apparent that a different set of controls maintain proliferation and destruction at a new level A significant difference is that the mechanisms (adrenal cortical hormones) for decreasing amounts of lymphatic tissue below a normal level are not present

The stress stimuli employed in all our studies indicate that they shorten the time of appearance and enhance the extent of alterations that occur in lymphatic tissue as a result of adrenalectomy alone[20] The lymphocytosis of stressed adrenalectomized animals appeared earlier and rose to higher levels but returned 24 hours after operation to the level characteristic of nonstressed adrenalectomized animals[20] There is an increase in the mitotic rate and in the size of the lymphatic organs of stressed adrenalectomized animal as well as in the number of their blood lymphocytes It should be emphasized that starvation is a special case for not only does the starvation act as a stress[18] but also the animal is dependent upon its own amino acid stores for synthesis of new protein Not only is there a great increase in blood lymphocytes in adrenalectomized starved animals as compared to lymphopenia in starved controls but also there is no decrease in lymphatic tissue nitrogen content[18] Indeed there is a slight increase above that found in intact controls It is also surprising that the number of mitotic figures is much greater in adrenalectomized starved animals than in intact starved animals Thus an animal may actually grow its lymphatic tissue in the absence of exogenous energy and amino acid sources for protein synthesis. The extent of hormonal control under these circumstances is surprising.

Relative Adrenocortical Insufficiency

Stress stimuli have been shown to induce a lymphocytic response not mediated by the pituitary adrenocortical hormonal axis. The sequential changes of circulating lymphocytes represent in numerical and temporal characteristics an inverse image of the curve of the lymphopenic response and have been termed the lymphocytotic response. The tendency of stress to enhance lymphocytopoiesis and lymphocytosis appears to be masked in intact animals by the growth moderating effect of adrenal cortical hormones also released by stress. Experiments were performed to delineate the comparative relationship existing between the *lymphopenic response mediated by pituitary adrenocortical hormones*.

Groups of adrenalectomized mice were given doses of cortisone varying from 50 μg to 0.5 mg,[9] that is, from doses that were ineffective in maintaining the lymphocytes within the *normal range to doses that produced and maintained* lymphopenia in the chronically treated adrenalectomized animals. Following a week of treatment, no further hormone was given and the animals were subjected *to daily stress by the administration of a sublethal dose of histamine*. Stress was performed daily for four days on all animals and at each interval lymphocyte counts were performed on those that survived the previous treatment. The blood *counts and the lymphocyte counts were obtained at four hour intervals for each* day. In each instance following the stress the lymphocytes rose to levels higher than those present at the start of the experiment and following a peak at 12 *hours, they returned approximately to the previous level at 24 hours*. At this time the animals were stressed again and the same procedure was followed. In each case the same lymphocytotic curve recurred with return to the previous level. The *increase of lymphocytes was least in the group that had been over*-treated, and greatest in the group that received the least pretreatment. The degree of lymphocytosis was inversely proportional to the amount of hormone treatment when the stress was constant. *The rate of kill of the adrenalectomized* cortisone-treated animals after successive stresses revealed as expected that there was a direct relationship between the amount of hormone given before *stress and the resistance to stress*. We have *interpreted this response as a relative* adrenocortical insufficiency that corresponds to phase I in the intact animal. This state exists when the amount of hormone available is insufficient to maintain lymphatic tissue and at the same time insufficient to meet the increased demands for utilization induced by stress. The increased movement of hormone from blood into the extravascular tissues results in a brief period in which the vascular and tissue compartments are not in equilibrium. It has been suggested that the lag phase occurs because hormone transfer from vascular to tissue *compartments proceeds more rapidly than in the opposite direction*.

Conversion products of cortisol (made by fibroblasts) then return to blood and come into equilibrium with the free compound so that these tissue products *are equal to amounts of free cortisol in blood*. When this state exists both blood concentrations of hormone and lymphocyte levels are in homeostasis. The concept of relative adrenocortical insufficiency implies that the rates of synthesis and release of corticosteroids may not be impaired but that the rates of inactivation at multiple sites are increased beyond the capacity for synthesis Thus lymphocytosis can occur when there is a normal or even increased output of adrenocortical steroids if hormone inactivation reduces the amount of free cortisol below statistically normal levels This appears to be the case in thyrotoxicosis[6] accompanied by a lymphocytosis where thyroid hormone enhances corticosteroid inactivation in the liver[25] by reduction of ring A

The normal diurnal variation of lymphocytes and eosinophils follows closely the fluctuations in adrenocortical steroid output[4] All leukocyte levels follow diurnal fluctuations that are dependent on adrenocortical secretion[4] The diurnal variation is most likely due to a fluctuation in the destruction of cells rather than being due solely to mitotic inhibition The variation during the day in rates of hormone output and inactivation regulate the changes in numbers of leukocytes In turn the levels of blood lymphocytes and eosinophils are indicators of the ratio of synthesis/inactivation of adrenocortical hormones

LYMPHOCYTE SIZE

Both the number and the size of mature lymphocytes of the blood are under corticosteroid hormonal control[6] In the adrenalectomized animal the lymphocytes increase in diameter[10] They increase to a size greater than that mean found for intact animals but do not undergo a progressive increase Actually this increase is because there is a smaller random distribution of sizes The largest cells are no greater than the largest cells of the intact animal[10]

It is obvious that in the absence of the adrenal cortex factors exist which bring about removal of lymphocytes since the rate of production and the rate

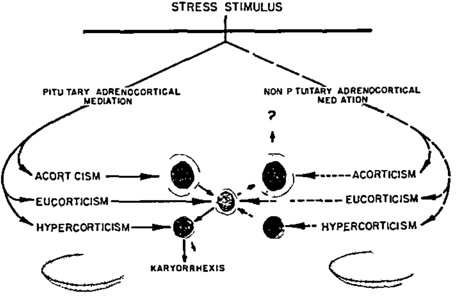


FIG 3 —The balance between pituitary adrenocortical mediation of lymphocyte size and nonpituitary adrenocortical mediation following a stress stimulus When the two are in balance the mean diameter of lymphocytes is characteristic of nonstressed intact individuals

of destruction must be equal in order that the constant level in the blood and tissues be maintained. Whereas adrenocortical secretions provide one mechanism by which lymphocytes undergo maturation and eventual death and removal, in the absence of this hormonal influence, other mechanisms tend to bring about their dissolution and death. Perhaps greater concentrations of cortisol per cell bring about karyorrhexis and cellular death whereas in the absence of cortisol, lymphocytes swell and undergo lysis.

Studies made in our laboratory and those made by Hernberg[18] have shown that the mean lymphocyte diameter is small in Cushing's syndrome; it is considerably larger than normal in patients having Addison's disease.[19] We have measured the lymphocyte diameters in patients with infectious mononucleosis, Addison's disease, and Cushing's syndrome, also in normal subjects given ACTH or an infusion of epinephrine.[9] In each instance when the blood steroid levels were higher than normal the mean lymphocyte diameters were smaller. When the lymphocytes increased in phase 1 in epinepherine-treated individuals the lymphocyte diameters increased too. In the cases of normal individuals given ACTH intravenously it was found that the reduction in lymphocyte population was almost entirely accounted for by a loss of small lymphocytes.[17] Thus we have the surprising situation of a marked reduction in the total number of lymphocytes but actually a greater mean cell diameter. This is because the larger lymphocytes (Type II Downey) are more resistant to the action of cortisol. Therefore as hormone levels increase, the total number of cells becomes smaller but the proportion of the large lymphocytes increases.

It should be emphasized that these changes in diameter occur for mature lymphocytes found in normal blood. We do not assume that large lymphocytes such as we have described in detail in our publications are immature cells. It appears from preliminary data that these large lymphocytes are cells that have an increased water content with little increase in protein. This would be an example of intracellular hydration in adrenal insufficiency.

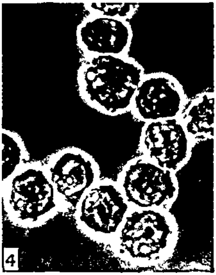
Dissolution of Lymphocytes

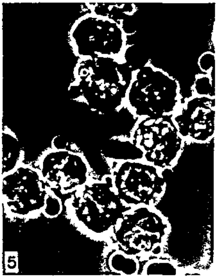
Changes in lymphatic tissues and organs following adrenocortical stimulation or hormone administration follow the same pattern as the changes in blood lymphocytes.[6] An enormous amount of information is available on this subject and can only briefly be summarized here. First of all cortisol and closely related lymphocytolytic hormones produce acute involution of thymi, lymph nodes, spleens, lymphatic nodules, and lymphatic aggregates. Acute involution as judged by loss of weight or loss of nitrogen content, is followed by a rapid return to normal values. When cortisol is present in excess amounts for longer periods of time it maintains a continued suppression of lymphatic tissue mass which, like blood lymphocytes, is rapidly reconstituted following the cessation of stimulation of endogenous hormone or of exogenous hormone supply. The hormone mediated effects following stressors also are similar to those found for the cells of the blood. Even the severe lymphatic organ depletion during starvation is stopped completely by adrenalectomy.

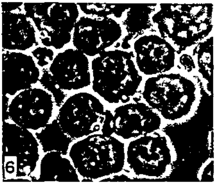
Similarly, lack of adrenocortical hormone supply influences lymphatic organs in the same way as blood lymphocytes. Following adrenalectomy, the mean lymphatic tissue and organ weight and nitrogen content increases until the fourth day following operation.[11] From this time until death, there is no further increment. When adrenalectomized animals are stressed, the rate of increment in lymphatic organ weight is faster than in operated animals alone, the organ weights are also greater than those following adrenalectomy. Thus, the organs manifest changes similar to those found during the lymphocytotic response in the blood.

CYTOLOGIC RESPONSES

The loss of lymphatic tissue mass has been attributed[14] to three important effects of cortisol and related hormones: (1) shedding of lymphocyte cytoplasm,







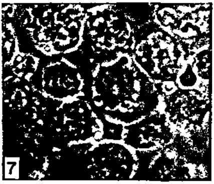


FIG. 4.—Phase microcinematographic pictures of AK ascites lymphoma cells 4 days after implantation. In vitro treatment with cortisol 0.00056 mμg per leukemic cell. 3 minutes after treatment.

FIG. 5.—Same cells as in figure 4. 20 minutes after treatment. Notice the budding of cells.

FIG. 6.—AK ascites lymphoma cells 4 days after implantation. In vitro treatment with 4 chloroprogesterone 0.0006 mμg per leukemic cell. 2½ minutes after treatment.

FIG. 7.—Same cells as in figure 6. 15 minutes after treatment. Karyorrhexis of lymphoma cells.

(2) pyknosis and karyorrhexis, and (3) inhibition of mitosis and heteroplasia Some authors[5] have questioned the phenomenon of cytoplasmic budding In our opinion it is unquestionably a direct action of cortisol on lymphocytes and has been recorded in vitro by time lapse phase microcinematography (figs 4 to 7) for normal and leukemic lymphocytes Cortisol apparently reduces the size of lymphocytes by inducing a shedding of cytoplasm but the nucleus also becomes smaller

The loss of cytoplasm and cell shrinkage probably proceeds with greater amounts of cortisol to karyorrhexis and death The medium sized and small lymphocytes of both blood and tissue are most susceptible to these hormone effects[14] Immature lymphocytes (reticular lymphocytes) of the lymphatic organs are extremely resistant to karyorrhexis and even show no reduction in cytoplasm[14] Later it was shown[5] that if animals were treated daily with large amounts of cortisol, lymphocytes became few in number in the lymphatic organs They remained pyknotic, but evidence of cellular destruction was not present after several days of treatment The reticular lymphocytes and reticular stroma cells were morphologically unaffected No mitosis or evidence of heteroplasia was present Upon stopping hormone treatment resumption of mitosis and apparently a return of cytoplasmic growth in pyknotic lymphocytes occurred

Many authors[5] have confirmed the initial report[14] that adrenocortical steroids inhibit lymphocytopoiesis Pfeiffer[23] has demonstrated that there is a log dose linear repression relationship between dose of cortisol and number of mitotic figures in the thymus This important quantitative procedure seems to establish once and for all the capacity of cortisol to inhibit mitosis and brings the action of this hormone in line with its other physiologic effects (dose response phenomena)

It is apparent that loss of lymphocytes from blood is at least in part related to the destruction of lymphocytes, since all lymphatic organs display loss of cells loss of protein, and loss of weight following cortisol administration[5] Microscopic evidence shows lymphocytic karyorrhexis and diminution in numbers of mitoses Therefore it has been suggested that acute lymphatic organ reduction is due to cellular destruction, and that more prolonged weight loss is due to inhibition of heteroplastic growth and mitosis of lymphocytes as well as to cellular destruction[5]

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Discussion of Regulation of Lymphocyte Production

W DAMESHEK Massive doses of ACTH and corticosteroids in acute leukemia often produce a remarkable remission From this it appears that massive doses of these steroids are antiproliferative, not only in lymphoid but also in granulocytic leukemias

In patients with large masses of lymphoid tissue in lymph nodes and spleen due to chronic lymphocytic leukemia or generalized lymphosarcomatosis, and in whom x ray therapy and the various alkylating agents are ineffective, the administration of massive doses of corticosteroids, 200–500 mg of prednisone daily, has produced dramatic responses Similar effects may be obtained in early cases of these lymphocytic proliferative diseases It would seem then that corticosteroids are antiproliferative agents—perhaps they are antimitotic in a broad sense they may be construed to be antimetabolic not specifically like the known antimetabolites but in a general way At the same time these agents may bring about an increase in bone marrow cells perhaps related to the fact that the lymphoid tissues are less active perhaps for some other reason that we don't know

These observations may have some relevance to observations on the effects of adrenocortical hormones on the immunologic state particularly in autoimmune disease Recent observations suggest that the immunologic state and the proliferative state that we know of as leukemia have several common denominators They may function in much the same way or at least have some relationships in common. The autoimmune state is a self replicating proliferation in which abnormal proteins are produced and the corticosteroids have a remarkable effect when given in large doses in these conditions Recently in line with these observations Schwartz in our laboratory demonstrated that the immune state may be affected by the use of a specific antimetabolite 6-mercaptopurine again bringing up the idea that the immunologic state is a proliferative state These observations may have some bearing on what has been stated here

J M YOFFEY The lymphocytes do undoubtedly form an extravascular pool which moves at an unknown speed through the tissues We can't assign any functions to it but it steadily moves back to the blood stream through the peripheral lymphatics

The question of large and small lymphocytes which arose out of Dr Craddock's paper highlights the importance of not translating results bodily from one animal to another At the same time I am somewhat surprised at his high proportion of large lymphocytes Our own data and that of Rous[1] on dogs some years ago gave a figure for large lymphocytes in the dog of about 5 to 7 per cent

A S GORDON Dr Dougherty have you considered the possibility of a peripheral antagonism between tetrahydrocortisol and cortisol acting at the level of the lymphatic tissues? Is it conceivable that the condition of the lymphatic tissues is dependent not only on the amount of cortisol but also on the quantity of naturally occurring and possibly antagonistically acting steroids related to the tetrahydro derivative? Have you ever injected tetrahydrocortisol by itself to see whether an effect is produced on the lymphocyte picture similar to that following adrenalectomy?

T DOUGHERTY We gave cortisol in doses equivalent to other hormones to see whether we got synergism or antagonism So far we haven't been able to show either Tetrahydril alone in normal or adrenalectomized animals produced no effect on the lymphocytes When we give tetrahydril plus cortisol we don't seem to block the action of cortisol. This doesn't mean there might not be some effect We have found only one steroid compound L, that has an effect on lymphatic tissue and this doesn't seem to be a peripheral antagonism

L S KELLY Dr Craddock does the specific activity for lymphocytes in blood eventually get almost as high as that of the granulocytes?

C G CRADDOCK The specific activity in the blood may reach levels approximating that in the lymph but not at the same time It reaches levels almost as high as the granulocytes but this does not persist

L S KELLY This is difficult to reconcile with the thymidine results where one finds a much smaller fraction of total lymphocytes than granulocytes labeled at the peak times The early rise of specific activity in lymph is presumably due to label in the larger cells but it is surprisingly high when one considers the small proportion of large cells

C G CRADDOCK Dr Kelly in the mongrel dog the percentage of cells capable of labeling with H^3 thymidine or incorporating P^{32} is around 15 to 20 per cent which is much higher than in the rat mouse or guinea pig

V P BOND In our experience with dogs the thoracic duct lymph contains only about 5 per cent labeled cells which is lower than that reported by Dr Craddock

I should like to comment on our studies on the fate of labeled cells in the peripheral blood at least some of which are derived from thoracic duct lymph In initial studies we noted large mononuclear cells in the peripheral blood of human beings that labeled in vitro with tritiated thymidine When we cannulated the thoracic ducts of dogs we found labeled medium and large lymphocytes within hours of thymidine injection Dr Yoffey and others have confirmed and reported on this at our meeting here In the human being we arbitrarily divided the types of cells found in the peripheral blood into five categories purely on a morphologic basis I shall say now only that all of the cells that label in vitro are large mononuclear cells We have not seen what we consider a small lymphocyte labeled after incubation for one hour in the test tube We find more of a variety of labeled cells in the peripheral blood of man than in animals and it is quite probable that all of the cells that we see labeled in the peripheral blood of animals are lymphocytic in character

Thymidine was injected into one of a pair of parabiotic rats while the connection between the two parabionts was clamped off After one hour the clamp was removed and the labeled cells allowed to travel from the donor to the recipient animal The recipient animals were sacrificed at various time intervals Both normal and irradiated recipients were used

Labeled cells have been found in all recipients In the peripheral blood of both donor and recipient labeled large and medium sized mononuclear cells were found Similar cells have been found in the bone *marrow spleen and lymph nodes of the* recipient In the marrow spleen and lymph nodes of the recipient large mononuclear cells were found which morphologically appeared different from those seen in the peripheral blood of donor or recipient More *of these morphologically different cells are* found in irradiated recipients than in normals particularly 24 hours following irradiation and the injection of thymidine into the donor We have not been able to identify these cells positively However they do appear to be distinctly different morphologically from those seen circulating in the peripheral blood No labeled cells in the recipient animals have been seen that could be identified positively as erythroid or myeloid precursors It is possible that these cells may be pluripotential in character and may represent transitional cells however it is equally possible that they may be wandering phagocytic cells

M M WINTROBE I would like to raise one point of the many that trouble me namely the reference to large medium and small lymphocytes I am not sure what people mean when they talk about these nor do I know their significance Many years ago Wiseman tried to classify lymphocytes as young mature and old more or less on the basis of size Such a classification has now been given up by most clinical hematologists because there are so many unknowns Do we know that there are three categories of lymphocytes or are those possibly gradations of the same cell?

In addition to the uncertainties which exist concerning the significance of the morphologic variations in lymphocytes which one admittedly sees it is very confusing to hear investigators refer to different types of lymphocytes without stating exactly what they mean This I am afraid is also a sad commentary on our methodology as well as on the state of our knowledge on the one hand we use analog computers and mathematical devices and we talk about things with seemingly great exactness on the other hand we talk so loosely about cells

C P LEBLOND I agree with Dr Wintrobe that whoever speaks of a cell should give quite precise operational criteria This was done in my paper since two slides with pictures of large medium and small lymphocytes were shown and each cell was *defined by size and appearance*

The destruction of lymphocytes by means of adrenocortical hormones is a way of dying that may occur in all tissues and may explain why these cells have to be continuously renewed These hormones may *have more subtle effects Thus Dr Kelly s* group showed a rather unexpected phenomenon When large lymphocytes were incubated in lymph there was a great deal of labeling with thymidine but when incubated in blood there was no such labeling Is it possible that there might be a higher concentration of adrenocortical hormone in blood than in lymph thus explaining why the cells divide less readily in the former than in the latter?

Another important point is the mechanism by which the cells die Dr Dougherty mentioned that he saw pyknosis and a loss of cytoplasm It is known that if you look at lymphocytes under the phase contrast microscope, you occasionally see a loss of cytoplasm, but I had the feeling that within a short time the cell involved was about to die Is not this cytoplasmic loss just another phenomenon, like pyknosis, indicating that the cells are going to die? In any case, would Dr Dougherty give us his opinion of the effect of adrenal hormones on lymphocytes in vitro? Can he produce their death in this manner?

T Dougherty I agree entirely with what Dr Wintrobe said, and want to emphasize the fact that I showed a frequency distribution which gave the size in microns of the cells He distinguished between the large, medium and small lymphocytes of the tissues as opposed to those of the blood, indicating the fact that one uses two quite different technics, and under one set of circumstances you might be dealing with a very different type of cell from a physiologic standpoint than you do in another

The phenomenon of loss of cytoplasm was described many years ago Our point has been that in the presence of certain adrenocortical hormones, the rate at which this event occurs is markedly enhanced It has been suggested there are other examples of a similar phenomenon This is one way that milk is secreted and we call it apocrine secretion In other words, it isn't a phenomenon that exists only in lymphocytes, it exists in other cells We have several miles of motion pictures of this and I wish that we had time to show this phenomenon going on and the remarkable rate at which steroid hormones pull off cytoplasm from cells which apparently go on living for four or five hours

L M Tocantins Isn't there some analogy between the sloughing of which you speak and the sloughing of platelets from megakaryocytes?

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Production and Life Span of Platelets

By T T Odell, Jr and Bonnie Anderson

THE EXPERIMENTAL RESULTS described here are an extension of previous labeling technics designed to study platelet production and life span, rather than to provide information about the actual regulation of platelet renewal about which very little is known Information on regulation of platelet production has been summarized by Cronkite[3]

In addition to previously described methods for determination of what has been called the life span of platelets in the peripheral blood[5, 6] we used another technic that is not subject to some of the criticisms brought against former methods A radioactive label, $Na_2S^{35}O_4$ was injected daily intraperitoneally into rats to maintain a constant daily pool of label in the animal (143 μc first injection, 100 μc thereafter) Injections were continued for a period longer than the presumed life span of the platelets Rats were killed at intervals and the radioactivity of the platelets separated from their blood was determined The results of two such experiments in rats are shown in figure 1 Platelet activity increased for five to six days after the initial isotope injection, and then leveled off The plateau indicates that new labeled platelets entering the circulation were merely replacing the labeled platelets that were being removed so that no further increase in total platelet radioactivity resulted The time from the initial injection to the plateau in this experiment includes the periods of labeling of platelets in the megakaryocytes their release to the blood stream and their circulation in the peripheral blood Other experiments have indicated that some labeling of platelets may occur in the blood stream but that most of the labeling takes place in the megakaryocytes[6] Since labeling of the most mature megakaryocytes and their disintegration into platelets probably occurs quite rapidly the period from isotope injection to plateau of the curve should provide a fairly accurate estimate of the total life span of platelets It is possible that platelets are held in the marrow for a short time after their origin but if so it is probably for only a few hours Therefore their time of residence in the blood stream equals their total life span as defined above, minus any time spent in storage before release into peripheral circulation Extrapolation of the curves in figure 1 back to the *abscissa suggests that 12 to 24 hours may be subtracted from the injection-to-*plateau time to obtain the circulation time of the platelets This gives a survival time of platelets in the blood of about 4 5 days, about the same as that found with various other methods Similarly, one can determine survival time of platelets in the blood from these curves by finding the percentage increase per day, and thereby the number of days required to completely replace the circulating population In these experiments the daily increases were about 22 per cent and 23 per cent, respectively, thus peripheral survival time was 4 5 or 4 3 days

This method avoids any prior handling of the platelets in contrast to platelet transfusion experiments and the results are more easily interpreted than in experiments in which the increase and decline in activity of platelets is measured

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From the Biology Division Oak Ridge National Laboratory Oak Ridge Tenn operated by Union Carbide Corporation for the U S Atomic Energy Commission

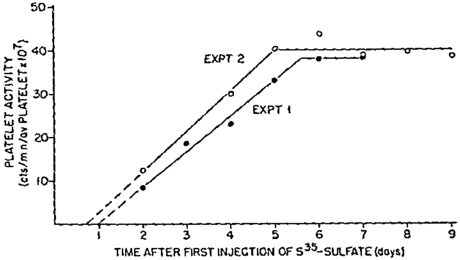

FIG 1 —Specific activity of platelets of rats injected daily with S^{35} labeled sulfate Each point represents the average of two animals

after a single injection of an isotope labeled compound The latter disadvantage does not, of course, apply to P^{32} labeled diisopropylfluorophosphonate (DFP^{32}), which is believed to be rapidly bound to circulating platelets rather than metabolically incorporated into them It has been shown that injected sulfate is incorporated in platelets into a mucopolysaccharide that is similar or identical to dom loss An exponential curve describing removal of platelets entirely by random loss can be drawn through the experimental points A curve depicting about 40 per cent to 50 per cent loss per day would fit the points reasonably well but a much greater or smaller percentage loss per day would not be consistent with the data

It is difficult to interpret data of the kind presented here in the case where both senescence and random loss are taking place Such a situation would be described by two exponential curves, one succeeding the other The point of change from one curve to the other would occur at the time when senescence began to make up part of the loss of labeled platelets In view of results obtained by other methods, it appears that random loss is not a major factor in removal of platelets from the circulatory system The results with DFP^{32} in man are a good example[4] If this is accepted our interpretation of the data obtained with regard to life span should be essentially correct

The accumulated information indicates removal of platelets from the circulatory system by senescence, with little or no random loss Yet this seems a somewhat unusual finding when one considers the function and metabolic state of platelets Besides taking part in the process of blood clotting platelets have some function in the maintenance of the integrity of capillary walls as seen in irradiation experiments and other thrombocytopenic states In view of their lack of a nucleus and their presumed lack of a very active metabolism it seems curious for them to undergo a maturation period of several days in the blood stream before use Perhaps a slow accumulation or loss of some material brings about their eventual extinction

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6 —— —— and Gude W D Uptake of radioactive sulfate by elements of the blood and the bone marrow of rats Am J Physiol *180* 491–494 1955

Discussion

E Adelson Tagging platelets in vivo we found random destruction 10 per cent levels being reached in seven to fourteen days We believe that coagulation is going on continually and platelets are actually used up in coagulation We find much shorter survivals perhaps three days when platelet transfusions are given to a man or animals with a hypercoagulable state e g postoperative On the other hand when platelets are transfused into the hypocoagulable individual in this case humans or animals on dicumarol the curve is more linear This we feel is due to a decreased utilization of platelets in coagulation platelets now die from senescence

Could some of the labeling Dr Odell observes be due to absorption onto platelets? Have any studies been done on platelets incubated *in vitro* with sodium sulfate? We found that inorganic sulfur does stick to the proteins It isn t incorporated into amino acids but it hooks on perhaps through a tyrosine group This was shown in the early work with insulin tagging Could this rise in platelet radioactivity simply be due to a rise in the plasma sodium sulfate levels? How does Dr Odell know that 100 μc a day are cleared? How does he know that it isn t a building up of the sodium sulfate level? Could it be simply that the plasma proteins are increasing in sulfate activity and absorb onto the platelets?

M Baldini Were platelets from polycythemic patients used in these experiments? In our experiments on survival of human platelets labeled with Cr^{51} we always found platelets from normal subjects were lost from the circulation in a linear fashion However using platelets from thrombocythemic patients (polycythemia vera or chronic granulocytic leukemia) the curves obtained were exponential

E Adelson Man and normal dogs

T T Odell I am very interested in these curves of random loss because this seems more sensible than what I have called senescence However Leeksma and Cohen[1] observed linear loss with DFP^{32} which is compatible with senescence rather than random loss

Sulfate goes only into mucopolysaccharides not into amino acids In extracting the sulfated mucopolysaccharides from platelets we have used either trypsin or sodium hydroxide which separate mucopolysaccharides from protein We removed the remaining particulate material by centrifugation Almost 100 per cent of the sulfate was in the supernate It was nondialyzable and was in the mucopolysaccharide fraction so I think that the sulfate was incorporated into mucopolysaccharide

In the experiments presented the platelets were washed two to three times with saline following separation from the blood and prior to counting We did serial plasma determinations and the plasma activity plotted out as a pretty good horizontal line indicating a constant pool size The plasma level the first day was below the final level

I prefer to call what we measure survival rather than life span We have been in the habit of calling it life span but if you called it survival you avoid presupposing either random loss or senescence If you speak of life span in any population you must recognize that all cells don t have exactly the same survival time

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Regulation of Cell Proliferation

By Edwin E. Osgood

THE AUTHOR'S DISCUSSION of the kinetics of cellular proliferation has appeared in a number of papers[2, 10, 11, 14, 16, 56, 58]. In this article all of his concepts on the subject will be presented in outline form, citing only such new references as were not cited in the previous papers, and making the treatment as general as possible.

The majority of cells of any series in multicellular organisms have measured finite life spans[12, 13, 17] and are incapable of division; these will be called "n cells" and their normal life spans "T." The cells of each series that are morphologically and biochemically most primitive do divide and maintain somatic genetic continuity. These will be called "alpha cells." Since the n cell is produced by the alpha cell, there must be a point of bifurcation, a division producing one alpha cell and one n cell; this will be called "(alpha, n) division." It leads to arithmetic increase in cell number. Some alpha cell divisions lead to production of two alpha cells[16]. These will be called "(alpha, 2 alpha) division." Each such division will double the total population of cells after (alpha, n) divisions have reached equilibrium. Each cell division of any type* adds one cell, not two, to the number present prior to the division.

Increased cell division occurs with the decrease from any cause of the mature n cells, as was effectively illustrated in the leukopheresis experiments of Craddock[4]. Also, vice versa, increase in mature n cells decreases cell division in the alpha cell. The typical example of this is the effect on erythrocyte production of blood transfusion to a normal individual. Shortening of the life span of the n cell leads to increased proliferation, even when the number of n cells is normal, as illustrated by compensated hemolytic anemia. Malignancy is characteristically associated with an increased proportion of dead cells. As Rose[53] has pointed out, fresh or living adult organ or tissue inhibits proliferation. Teir[57] has shown that old or dead adult organ or tissue stimulates proliferation of the corresponding tissue. In tissue culture, fresh, normal serum is inhibitory[7, 15]; old or diluted fresh serum permits growth. In man, fresh transfusions inhibit leukocytes; bank blood does not[60]. Unstable specific inhibitors have been demonstrated for kidney and liver by Saetren[55]. Many stable, less specific stimulators of cell proliferation are known, e.g., hormones and erythropoietins[3]. Malignant proliferation is characterized by a constant decreased ratio n/alpha. All other proliferations are characterized by a transient decreased ratio of n/alpha which is followed by an overcompensation with a transient increased n/alpha ratio.

Growth of unicellular organisms is of the semilogarithmic type where both cells resulting from a division are capable of dividing again. Normal growth in

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* Rare exceptions are multipolar divisions.

mammals is of the log log type,[20] not semilogarithmic Increase in cell number, when only the most mature cell of a series is missing, is arithmetic[16, 17] This again is well illustrated in Craddock's graphs[4] There is usually an overshoot with a damped oscillation in return to the steady state after recovery from perturbations The initial rate of DNA labeling far exceeds the rate of net new cell production[1, 16]

To preserve the characteristic life spans for the n cells of each series and the factors that regulate their growth response, transmission must be by the genetic material of the cell, both through the gametes to the progeny and from the fertilized ovum to the somatic cells In other words, the ultimate control of the growth, both of the soma and the species, resides in the genetic material of the cell[5, 56]

Concept of Growth Regulation

Primary regulation[2, 11, 14, 16, 24, 39, 40] is general and applies to each cell series It consists of a pair of specific feedback inhibitors of cell division produced by the n cell of that series and affecting only the alpha cell of that series

Mathematically for each series in each species, cells with the properties described for the alpha and n cell are necessary, and the n cell must be derived from the alpha cell In other words, there must be a point of separation, in (alpha, n) division Any divisions that may occur in the n cell (n, n divisions) are lost to somatic genetic continuity and necessitate a corresponding decrease in the division rate of the alpha cell to maintain the constant n/alpha ratio observed in the steady state in the healthy adult The alpha cell, which is a direct descendant in evolutionary time from the unicellular organism, retains the capacity of the unicellular organism for (alpha, 2 alpha) divisions, but with the coming of the multicellular organisms, it developed the capacity for (alpha n) divisions It is this latter type of division that occurs almost exclusively in the healthy adult to maintain the steady state

Every (alpha, 2 alpha) division ultimately not only doubles the population of alpha cells but also doubles the n cells and their products Even 20 such divisions would increase the total mass by a factor of a million Such divisions are therefore exceedingly rare in the healthy adult occurring only frequently enough to replace alpha cell loss The corresponding n cell early in differentiation produces a specific unstable inhibitor of this (alpha, 2 alpha) type of multiplication, which is maintained at an equilibrium concentration in the steady state Later in differentiation of the n cell a specific unstable inhibitor of the (alpha, n) type of division is produced, reaching an equilibrium concentration which in conjunction with the fixed upper limit of n cell life span maintains the normal n/alpha ratio observed in the steady state for each cell series in each species Prevention of feedback, removal of mature stages, or early death of the n cell leads to a decrease in the equilibrium concentration of inhibitor

These n cell-produced inhibitors have analogs in the inducers, evocators and organizers of embryonic development, which are also humoral substances produced by nonendocrine cells influencing growth[16] Any alteration of the genetic material in the somatic alpha cell of a series which leads to early death of the corresponding n cell may result in a spectrum of neoplasms of corresponding cellular type, from the most acute, rapidly progressive malignant tumor to the most slowly growing benign tumor, depending on how early in the life span of the n cell death occurs. Every alpha cell division gives some risk of such a genetic alteration. This may be a true mutation, a gene deletion, a chromosome change, or an alteration in a plasma gene, but it must be genetic and it must be in the alpha cell. A lowered local or systemic concentration of inhibitor increases the likelihood that such a change will be expressed as a neoplasm, since adjacent normal cells producing inhibitor would otherwise tend to keep such growth in check.

Each alpha cell for growth requires a suitable environment. The importance of an ecology of cells and microenvironments are well documented both in tissue culture[23, 74] and in the body.[53] Differentiation probably results from sequential inhibition of gene expression by similar feedback mechanisms,[54] but it is not reversible at the n cell stage. What has been called dedifferentiation and maturation arrest, actually represents early death of the n cell[16, 17] with a resultant differential rate of multiplication by the alpha cells.

The predisposing factors for uncontrolled proliferation—in other words malignancy[18]—are factors increasing the risk of genetic change such as hyperplasia or mutagens, or factors tending to lower the concentration of inhibitor reaching the alpha cell, such as hypoplasia isolation, or protection from diffusion. All known carcinogens fall in one or the other or both of these categories.

Secondary regulation is a correlation mechanism[3, 9] that tends to control the microenvironment of the alpha cell. The same mechanism usually affects several cell series. If chemical or humoral, the agent is produced by cells not directly related to the cells acted on. The control may be a result of the physical and chemical environment of the organism. The action may be variable on different cell types and tends to result in hyperplasia hypersecretion or hypertrophy on the one hand or hypoplasia, hypo-secretion or atrophy on the other. The secondary controls represent the fine adjustments that determine the microenvironment of the alpha cell, while the primary controls represent the coarse adjustments that determine the type and potential rate of multiplication of the alpha cell between fixed limits of zero and the maximum capacity for the species and cell series.

Examples of the secondary controls include hormones, erythropoietins, Menkin factors, adsorbing surfaces, neurogenic controls histamine, antigen antibody reactions, water, vitamins, electrolytes, nutrients, temperature oxygen tension, CO_2 tension, pH, oxidation reduction potential, and local and systemic blood and lymph flow. According to the author's concepts, these secondary regulators influence neoplasia only as their action results in hyperplasia hypoplasia or alterations in diffusion rate, in other words, only as they influence the risk of change in the genetic material of the alpha cell or the level of inhibitors in the microenvironment of the alpha cell.

In all studies of cell growth, it is important to distinguish between the steady state condition[17] in the healthy adult of the species and proliferation under conditions in which the inhibitor concentration reaching the receptor locus in the alpha cell is reduced. We should recognize that there is a maximum capacity for (alpha, 2 alpha) division and for (alpha, n) division inherent in the genetic make-up of the alpha cell. We should also clearly distinguish between the transient alterations in inhibitor level that tend to correct themselves and the neoplastic reductions in inhibitor concentration that could theoretically only be corrected by a life time supply of either inhibitor or, much more promising, the missing factor necessary for the normal life span of the n cells. We must also remember that for each cell series in each species evolution has probably provided the optimal range of life spans for the n cell and the range of generation times for the alpha cell[5] both for (alpha, 2 alpha) and (alpha, n) divisions for that specific cell type in the specific species at that specific stage of growth and for the type of environment to which the species has been most recently required to adapt, in terms of evolutionary history. There will be wide variations in generation time and n cell life span inherent in different alpha cells of the same tissue and species, in the same individual and between individuals, but for each of these factors there will be a mean value as well as a probability distribution.[50, 51] Unfortunately, this makes it impossible to transfer data from one to another species or tissue or even from one to another growth stage within a species and tissue.

IMPLICATIONS

From these considerations the primary unit of growth is the expressible gene complement of the alpha cell.[6] Each gene complement not only will determine the protoplasmic mass and composition of the alpha cell but also will carry with it a fixed number and composition of n cells and extracellular fluid and cell products such as collagen, bone, cartilage and other extracellular compounds.

The secondary controls permitting the rate and type of proliferation (alpha, 2 alpha or alpha, n) is determined by the concentration of the respective inhibitor reaching the receptor locus of the alpha cell. This local concentration of inhibitor is in turn regulated by the ratio $t_i/T_i \times n'/n$, where T_i is the normal life span of the n cell up to the point of full production of the respective inhibitor and t_i is the actual mean effective life span. The actual mean number of n cells within diffusion range of the alpha cells is n′, and n is the corresponding number of n cells in the normal.

In all conditions other than neoplasia, t_i approaches T_i as an upper limit. In neoplasia, the upper limit of t_i is less than T_i. In the normal adult steady state the ratio is 1.0 and corresponds to the normal equilibrium concentration of the respective inhibitor. Note that the decay plus excretion rate of the inhibitor must equal the production rate.

In the healthy adult (alpha, 2 alpha) divisions must be exceedingly rare, only enough to replace alpha cell loss, for each such division of all alpha cells doubles the population of that series and its products. Only about 32 such divisions are necessary for all fetal growth and 4 or 5 for all postnatal growth.[6] The finite life span necessitates many (alpha, n) divisions per alpha cell in the healthy adult during one life span of the n cell, about 183 for the erythrocyte.[15] Because they are separated in time only one of these per alpha cell may be counted even with the use of mitostatics such as colchicine. Only an alpha cell can start a colony in tissue culture,[16] or a transplant or a tumor. All n cells initially present are dead by time T.

Aging, as pointed out by Failla,[6] is the result of the accumulation of minor genetic alterations in the alpha cell, leading to decreased efficiency in the production of essential compounds by the n cells Lethal alterations in the alpha cell are readily corrected with replacements by (alpha, 2 alpha) divisions

Genetic loss[3] without shortening of the life span of the n cell would neither explain the observed n/alpha ratios in malignancy nor the prevalence and varied enzyme content of malignant tumors Rare neoplasms due to loss of the gene for the receptor site or for inhibitor production are compatible with these concepts, as are neoplasms due to extracellular factors necessary for the normal life span of the n cell Pernicious anemia is an example of the latter

The essential feature of this concept[18] is the shortening of the life span of the n cell due to alteration of the genetic material of the alpha cell Only the replacement of the normally gene-produced missing factors necessary for a normal life span of the n cell, in the sense that B_{12} does this for the pernicious anemia alpha cell, would lead to a real control of a malignant neoplasm

In conclusion, an attempt has been made to present a completely general outline of the essential facts of cell proliferation, to summarize in concise sequence the concepts of a mean mathematical model, neglecting probability factors that would explain all these essentials, and to discuss the implications of the roles of the primary and secondary controls, as regards normal and neoplastic cell growth, differentiation, and aging

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Discussion

H Warner Dr Osgood it is your contention that regulation of hemic cells occurs only through regulation of the stem cell The evidence for this as I understand is that in the steady state the relative number of each cell type in the marrow is fixed To adequately analyze a regulating system it is necessary to disturb that system transiently in some way and observe how it returns to an equilibrium Not being a hematologist I know little about bone marrow behavior However is it not true that if this system is disturbed such as in infection the differential count does not remain constant? If true it seems to me this would argue against regulation through the alpha cell alone but would argue for direct regulation of the rate of production of other cell types down the line as well

E E Osgood I make no attempt to present the evidence here The evidence is presented in some forty of our previous papers the references to which are given in our paper The DNA labeling technics however do not necessitate any changes in the hypothesis which I presented

Section V. Kinetics of the Regulation of Red Cell Production

R. A. Good, *Chairman*

Observations on the Regulation of Erythropoiesis and on Cellular Dynamics by Fe^{59} Autoradiography

By EDWARD L ALPEN AND DORIS CRANMORE

IN A PREVIOUS REPORT on our earlier results with Fe^{59} in the study of bone marrow metabolism[1] the authors presented some of their findings concerning the kinetics of maturation of the erythroid elements as well as information on the kinetics of distribution of inorganic iron Certain of the findings that were not completely understood then, have been further examined and several technics described there have been further developed

The most troublesome aspect of our early work was in the finding that very large increases in daily output of erythrocytes could be effected with only small if any, changes in the various parameters of maturation dynamics that were measured In this report further data bearing on this problem and our concept of a possible model for regulation of erythropoiesis are presented Because the subject of technics used in the study of cellular kinetics is pertinent the utility of Fe^{59} as an autoradiographic tracer for bone marrow studies is also discussed

METHODS

All the following studies have been carried out on young adult mongrel dogs maintained on a laboratory diet after veterinary care for elimination of parasitic infestation and disease The dogs were anesthetized with sodium pentothal and bone marrow samples were withdrawn from their ribs or ischial crests into a 2 ml syringe containing autologous heparinized plasma All procedures* on the

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From the U S Naval Radiological Defense Laboratory San Francisco Calif

* Cytologic Criteria of Differentiation *Erythroblast* Cell of 20–30 μ diameter oval to round with a large nucleus 0 7 to 0 8 of cell diameter Cytoplasm strongly basophilic giving a characteristic prussian blue color This is the most important differential characteristic of the early erythroid cell Chromatin of nucleus is reasonably finely reticulated with only slight evidence of clumping Nucleoli present but not outstanding *Pronormoblast* 15–20 μ diameter with relatively more cytoplasm Cytoplasm still blue Nucleus has more distinct chromatin clumping and gives the impression of being more darkly staining than in the erythroblast *Normoblast* Cells slightly smaller to much smaller than pronormoblast Nucleus now has the characteristic chromatin pattern of late erythroid cells Cytoplasm now becoming polychromatic due to presence of increasing quantities of hemoglobin The *polychromatic* normoblasts are those with blue grey to grey cytoplasm The *orthochromatic* normoblast has a cytoplasm identical in color to that of a mature erythrocyte and usually has a compact dense pyknotic nucleus

marrow or blood samples have been exhaustively described.[3] Autoradiographic technics, as well as the technics of grain counting and determination of per cent positivity, are also described. Radioactivity measurements on all samples were made with an end window scintillation counter on samples prepared in cupped planchets, one inch in diameter.

RESULTS

Cellular Dynamics and Regulation of Erythropoiesis

Further testing of the technic of estimating the mean generation time of the erythroblast has shown it to be particularly valuable and very precise. As the erythroblast is the earliest cell type in the erythron that incorporates significant amounts of Fe^{59}, the kinetics for disappearance of the label from this cell type is undisturbed by the entry of labeled cells from the maturation of a precursor. In this situation we can evaluate the average generation time of an erythroblast from the time required to halve the median or mean grain count. In order for the technic to be valid, certain conditions must be satisfied: (a) The label must leave a single cell by the process of division only, i.e., there must be no label elution. (b) Maturation without division must not preferentially remove a given class of cells with high or low grain count. (c) The general shape of the distribution curve for grain counts must not change, i.e., there should be no preferential division of either high grain count or low grain count cells. As an extension of the last requirement, a very high proportion of the total cell population must be labeled at zero time in order to avoid the bias introduced by the presence of two populations, one incorporating Fe^{59} and one not. It is assumed on the basis of accumulated experience of many investigators that intracellular iron is retained, but all other conditions may be tested.

Because of the very high grain counts achieved by the erythroblast, the appearance of any significant proportion of them as erythrocytes after no divisions or only one division would certainly be detectable. A small number (less than 5 per cent) actually do follow this pathway, as will be discussed later.

Figure 1 is presented to show the general nature of the grain count distributions after a single injection of Fe^{59}. There is no appreciable alteration in the shape of this curve as time advances, indicating that condition (c) is well satisfied. The use of the median as the measure of central tendency further helps to smooth out irregularities and to prevent the small number of very high grain count cells, which are invariably present, from biasing the measurement.

Figures 2 and 3 are presentations of the erythroblast grain count data as outlined above. The median grain count is plotted logarithmically as a function of time. The values during the incorporation period when radioactivity is still present in the plasma are ignored. The half times or generation times thus measured are all in the range of 17 to 20 hours for the control animals; similarly, the bled animals have mean generation times of 17 to 18 hours. This values is to be compared with 20 to 24 hours, derived by Lajtha[4] for the human erythroblast (his pronormoblast). On the basis of this evidence, we must accept that the *increased rate of erythropoiesis is not mediated by shortening the generation time* for this precursor cell. Furthermore, although it is impossible to describe the generation

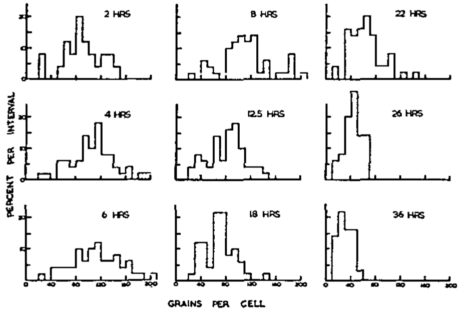

Fig 1 —Grain count distribution for erythroblasts at various times after injection of Fe^{59} Fifty cells were counted on each sample

times for the pronormoblast or for the normoblast in terms of a simple mathematical function, because of the complex pattern produced by the passage from one cell type to the next, the subjective judgement can be made that there is no remarkable change in the rate of fall of median grain counts for either the pronormoblast or normoblast

Further examination of the methods of grain-count extremes previously outlined[1] has not yet yielded further information on cell generation times in dogs which have been bled However, examination of peripheral blood smears that have been supravitally stained with brilliant cresyl blue, has allowed us to make an independent confirmation of the average time spent in the bone marrow by the reticulocyte in bled and control animals The figure that we obtained by direct grain-counting on marrow reticulocytes[1] in unbled animals was 22 hours When peripheral erythrocyte grain counts are made on preparations in which reticulocytes are identifiable, a few of these cells are lightly labeled at very early times The first peak of strong activity occurs at 24 hours after injection of the label in either bled or control animals and represents the mean duration in the bone marrow for those reticulocytes that were directly labeled at the time of isotope injection The very early appearance of active cells in peripheral blood is the result of the direct labeling of the blood reticulocytes Another major peak of reticulocyte labeling occurs at 48 hours, caused by the arrival of the bulk of normoblasts labeled late

The small group of directly labeled blood reticulocytes of low grain count disappear in about 30 hours after isotope administration indicating that these cells are converted into orthochromatic erythrocytes in about that time interval No significant differences in maturation time for either the marrow or the blood reticulocyte is observed after bleeding

The remaining point bearing on the question of regulation of production of erythrocytes is the evidence provided by mitotic index determinations In this regard, it is essential to point out that one must differentiate by cell type when determining mitotic index in the nucleated erythron, because the cell cycle is not of constant length for all types From table 1, it is clear that no significant alteration in mitotic rates is produced by bleeding As there are no marked differences in the relative proportions of the various cell types, the over all mitotic index is also unchanged

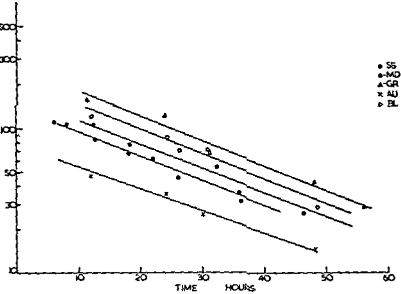

Fig 2 —Median grain count (+IVE cells) of erythroblasts plotted logarithmically against time The generation time for this cell type is estimated by reading the time for half reduction of the median grain count The data shown are for five control animals Animal GR (Δ) was purposefully infused for a prolonged period of time (six hours) to check the influence of plasma clearance time of radioactivity on the estimate of generation time

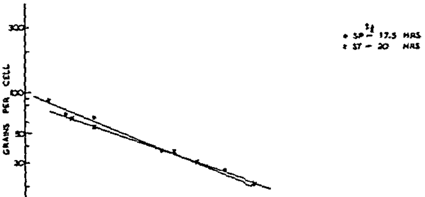

Fig 3 —Median grain count (+IVE cells) for erythroblasts of two bled dogs SP (●) was bled 40 ml /kg , and ST (×) was bled 100 ml /kg

TABLE I.—*Mitotic Index for the Erythroid Cell Types in Normal and Bled Dogs**

	Erythroblast		Pronormoblast		Normoblast	
	mitoses/total	%	mitoses total	%	mitoses total	%
Total measurements on 8 normal dogs	128/1420	9.0	416/10642	3.9	380/14341	2.6
Total measurements on 4 bled dogs	110/1308	8.0	362/9[illegible]0	3.7	295/8630	3.4

* It is difficult to identify bone marrow cells in prophase and telophase so that one should interpret the above data as referring only to the percentage of cells in metaphase and anaphase.

The pattern we have observed is that under conditions of severe bleeding no significant changes can be found in the several parameters of maturation dynamics measured. If we recapitulate all the observations made here as well as those referring to the questions of numbers of divisions occurring in the erythron reported in our previous paper[1] we see that (a) The average number of cell divisions that must occur for the nucleated bone marrow to produce the observed number of erythrocytes was shown to be 1.3 for unbled dogs 1.1 for dogs bled 40 ml./Kg. and 1.3 for dogs bled 100 ml./Kg. This value which is merely the logarithmic ratio of the measured number of nucleated erythroid cells to the measured number of mature erythrocytes produced is heavily weighted by the large number of late forms that do not divide at all or that divide only once. (b) The most likely number of divisions that an erythroblast undergoes as measured by the relationship between the grain count of the erythroblast and the grain count of the peripheral red cell was determined to be 2.3 for control animals and 2.2 and 2.4 for the two types of bled animals. This value represents a truer picture of the number of divisions actually occurring. It would indicate that approximately half the cells divide three times and half only twice. The missed division in the latter half is probably in the late polychromatic-early orthochromatic stage. Many of these cells are seen dividing in marrow smears but it is unlikely that all do. (c) As outlined earlier the generation time for the erythroblast remains at 18 hours in spite of severe bleeding. (d) No significant alteration in over all erythrocyte maturation time can be attributed to accelerated ripening of the reticulocyte. (e) Mitotic indices for three major cell type in the erythroid series are not altered by bleeding.

At this point we see no answer to the question of how the bone marrow responds to a proliferative stress. However in figures 4a and 4b a major difference is seen between bled and control animals. These data are a plot against time of the proportion of each of the three major cell types that are positively labeled. It can be seen that whereas 100 per cent of the erythroblasts in the control group are still labeled at 24 hours after isotope injection the percentage in the bled group is less than 10 per cent. This large difference can be explained only to a very small extent on the basis of the more rapid removal of radioactive iron from plasma with a decrease in labeling time. In the most extreme case this increased rate of plasma clearance could not account for more than a two to three hour shortening of the labeling period. In the control animals three half times would be approximately five hours while in the bled animals three half times would be about two hours. We have already demonstrated that the rate of decrease in the grain count over *positive* cells is not significantly different in the two groups. Hence, the large difference in rate of change of per cent positivity can only be attributed to *the entry of significantly larger numbers of unlabeled cells into the labeled erythroblast compartment in the bled animals.* The basis for our model for regulation of proliferative rates is based on this interpretation of the data.

Kindred[3] first pointed out, and we subsequently reaffirmed it from our own data,[1] that if one calculated the mean sojourn of a cell in the nucleated erythron from the mitotic index and any reasonable estimate of mitotic duration, the result was in marked disagreement with the known time that a cell spends in the bone marrow. Kindred stated that a proportion of the daily proliferation of the erythroid elements was supplied from the "hemocytoblast." The accumulating evidence from our study is in support of his hypothesis and also directs attention to this mechanism as the one primarily responsible for the proliferative response of the bone marrow to bleeding. When a requirement for increased production of erythrocytes is imposed, a rapid increase in the rate of differentiation of the

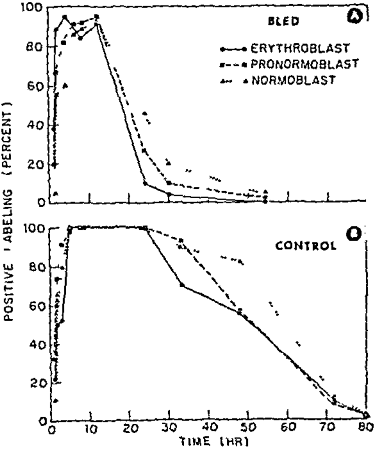

FIG. 4.—Per cent positivity estimates from marrow smears at various times after injection of Fe^{59}. *Figure 4A is for bled animals and 4B for control animals.* (Reproduced by permission from Ann. New York Acad. Sc.)

multipotential stem cell is effected, with a consequent enlargement of the nucleated erythron and an increased rate of red cell production

The major points in support of this hypothesis are the observations that the directly measured generation time for the erythroblast is unchanged after bleeding, and the per cent positivity" curves indicate an influx of large numbers of unlabeled cells into the erythroblast compartment It should be noted that in the bled animals, 10 to 15 per cent of the cells morphologically classified as erythroblasts fail to incorporate Fe^{59}, while in the control series only 2 to 3 per cent fail to do so In the normal animal, if a small proportion of new cells continuously enters the erythroblast compartment, then one should be able to detect them, provided the acquiring of morphologic characteristics of the erythroblast is more rapid than the acquiring of the ability to incorporate iron We feel this is the case and that the increased percentage of unlabeled cells in the bled animal reflects the influx of a larger proportion of freshly differentiated erythroblasts Although the biochemical mechanism underlying differentiation is not known, apparently these cells acquire their morphologic characteristics before they acquire the capability to incorporate iron

A necessary requirement of any regulatory model is that it shall be sensitive to the decreasing need for cell production and be capable of reverting to the normal prebleeding state Preferably one wishes to see a system that does not require the elaboration of some special anti hormone or negative regulator for this purpose An observation first made by Lajtha and Suit [5] and subsequently confirmed by us, allows for the formulation of a possible governing mechanism Lajtha and Suit remarked that a small proportion of erythrocytes produced after a single injection of Fe^{59} have grain counts that could be obtained only if these cells had arrived in the peripheral blood after a single division of the erythroblast In a small proportion of cells, what they call the basophilic normoblast (our pronormoblast) apparently proceeds without further division through the stages of maturation and hemoglobinization This process may represent the result of an erythroblast division where one or both of the daughter cells are incapable of further division We have called this process terminal division and outlined it in figure 5, it is to be compared with normal erythroblast division wherein the cell line is sustained (fig 5) In both figures it is to be noted that we have not indicated divisions occurring after the early normoblast stage Marrow smear evidence indicates that some further divisions occur, but the frequency and importance of the process is not known Division in the late normoblast stage is peculiar to the dog and is not often observed in man

The terminal division scheme results in the elimination of a single erythroblast This normally is replaced to maintain the steady state concentration of cells, but in the case of the hyperplastic bone marrow where accelerated production is no longer necessary, this mechanism can provide the means whereby the expanded marrow can be reduced to normal limits

In figure 6 is pictorially presented the proposed scheme of erythropoietic regulation In the normal animal in a steady state, a small proportion of the erythroblasts are removed daily by the terminal division process or an alternate leak The cells which are removed in the resting steady state are replaced at a rate r_1 from some multipotential stem cell, and rate r_1 is equal to the rate r_2 at which

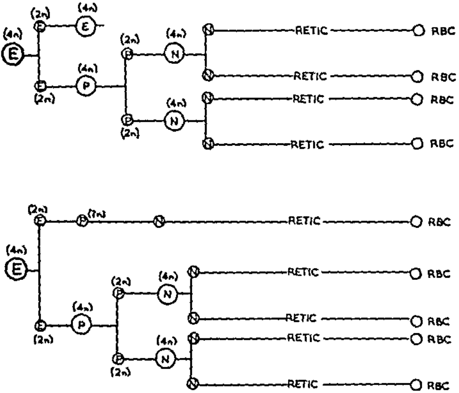

FIG 5 —Schematic representation of normal (*top*) and terminal erythroblast division In the normal division the cell line is sustained by the nondifferentiation of one daughter cell while in the terminal scheme the erythroblast is lost *Divisions in the normoblast stage are not indicated but they certainly occur in the dog in some proportion of cells*

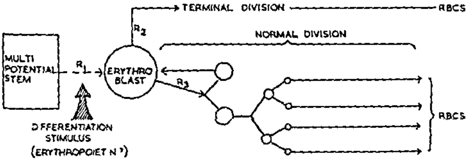

FIG 6 —A proposed model for regulation of erythropoiesis The nature of the multipotential stem is not indicated

the erythroblasts are being lost In the steady state $r_1 = r_2$ It is assumed that r_2 is controlled as a first order process that is a constant fraction of the erythroblasts present are lost per unit time After bleeding the primary erythropoietic stimulus (anoxia, blood volume, or both) causes the elaboration of a humoral substance capable of increasing the rate of differentiation (r_1) from the multipotential stem into the erythron When the blood level of the humoral regulator is stabilized, a new steady state will be achieved, with r_1 again equal to r_2 When the primary stimulus is removed by the return of the blood volume to normal levels the humoral differentiation stimulus is reduced, and r_1, the rate of differentiation, returns toward normal As the erythroblast compartment is expanded, r_2, which we have formulated as a first order process is now higher than r_1, and cells are removed in excess of new production until the pre existing steady state at normal levels is reached

The nature of the leak ’ process that leads to elimination of erythroblasts is not critical to the argument We cannot exclude other processes that may serve the same function Erythroblasts may divide and produce two cells incapable of further division, or a certain proportion of erythroblasts may, in all conditions normal or hyperplastic undergo cell death or be otherwise eliminated It is necessary only that normal bone marrow have a small cellular attrition rate and to postulate that a leak exists and that the absolute magnitude of this leak is proportional to the number of cells present

The Role of Erythropoietin

From the process described above we see that the humoral regulatory substance, presumably erythropoietin has as its primary function the directing of differentiation of the multipotential stem cell, determining the rate at which stem cells are converted into erythroid precursors From the model, it is also apparent that erythropoietin must exert its regulatory function even in the normal state That is to say, r_1, the rate of differentiation of new stem cells is determined by the level of erythropoietin in the normal steady state

How pure the actions of erythropoietin are remains to be seen Careful analysis of the effects of erythropoietin on cell generation times and DNA synthesis rates is necessary Some evidence is already available, however, to indicate that erythropoietin acts also on the later forms of the erythroid series In the first place the model described above would predict that at steady state conditions after a bleeding stress, the differential distribution of cell types would be unchanged This is nearly but not completely true Table 2 presents the analysis of total marrow cellularity and differential distribution before and after bleeding The marrow cellularity was determined by the method of Suit[7]

These numbers do not have a high degree of precision but in any case it is clear that bleeding is causing a disproportionate increase in the earlier forms This observation would be consistent with the proposed model if, at the time of measurement, the marrow were still expanding and had not yet achieved a steady state

A second bit of evidence bearing on this question is to be seen in the peripheral

TABLE 2 —*Marrow Cellularity and Distribution by Cell Types in Bled and Control Dogs*

	Normal		Bled 40 ml./Kg		Bled 100 ml. Kg	
	Number $\times 10^{-9}$ Kg	Ratio Pro = 1	Number $\times 10^{-9}$ Kg	Ratio Pro = 1	Number $\times 10^{-9}$ Kg	Ratio Pro = 1
Erythroblast	0 13	0 09	0 4	0 13	2 2	0 18
Pronormoblast	*1 50*	*1 00*	*3 1*	*1 00*	*12 0*	*1 00*
Normoblast	2 20	1 50	4 0	1 30	10 0	0 80
Reticulocyte	3 30	2 20	6 0	1 50	22 0	1 80

radioiron appearance curve. After bleeding, a small but significant decrease in the time for one-half maximum peripheral appearance has been reported by us, but is certainly not a novel finding. For example, for the same animals for which the marrow cellularity is presented in table 2, the time for one half maximum iron incorporation ranges from 2.8 days in the control animals, to 2.4 days in the less severely bled, to 1.6 days in the heavily bled. Because there is less than 0.2 days difference in the labeling time for the most extreme case, there must be a significantly shorter time in the marrow for the developing cells of the bled animals. This small difference may be accounted for by shifts in the marrow differential distribution that are not detected by our methods. For example, if the reticulocyte number were relatively increased there would be an apparent shortening in the time for half the activity to appear in peripheral blood.

None of these observations indicates changes in the marrow dynamics compatible with the tremendously increased output of erythrocytes, and it is worth commenting at this point that it is unlikely that the marrow cells are capable of drastic changes in maturation times that would account for the tremendous range of performance of which the marrow is capable. However, careful investigative work is indicated to evaluate these changes. In all the studies reported here no measurements were made earlier than several days after the first bleeding, and it is possible that investigation of the early postbleeding times will reveal some effects on the maturation time during the period of maximum marrow expansion. An alternative to the stem cell providing the source of increased cellularity during the hyperplastic stage is for the erythroblast itself to respond by increased geometric division without maturation, thus increasing the marrow cellularity. This hypothesis requires, however, that the increase in marrow cellularity should stop before the blood volume is restored. What the regulatory method would be we cannot at this time visualize.

Any treatment of the subject of the actions of erythropoietin would be incomplete without discussing the observations of Schroeder, Gurney, and Wickman[6] that erythropoietin causes dramatic increases in the rate of radioactive iron utilization by bone marrow cells in tissue culture. They observed that the radioactivity in bone marrow cells suspended in anemic plasma was as much as four times greater than that of cells suspended in normal plasma. Such an observation left unchallenged lends great weight to the arguments for the direct action of erythropoietin. Alpen and Lajtha[7] have repeated these experiments with urinary erythropoietin kindly provided by Dr. Van Dyke of the Donner Laboratory in Berkeley, and have found that in human bone marrow culture only insignificant increases in iron incorporation can be demonstrated with massive quantities of the hormone. The discrepancy between these data and the observations of Schroeder et al. await clarification, but the critical deficiency in the latter workers' report lies in the lack of knowledge of the final specific activity of the administered isotope when plasmas likely to have widely different plasma iron concentrations are used.

Fe^{59} as a Tool in Bone Marrow Investigation

Radioactive iron is, at best, a difficult isotope to use in autoradiography. It lacks the exquisite resolution of the tritium labeled compound; it lacks the long half life and good resolution of carbon^{14}. But in spite of these disadvantages it is still unsurpassed for use in studying the kinetics of the erythroid series It labels every cell morphologically identifiable as an erythrocyte precursor, and fortunately labels them at levels roughly corresponding to the position of the cell in the development line Of paramount importance, the nonexchangeability of the isotope, once incorporated, still stands as an undisputed fact

On the question of resolution, it must be borne in mind that very high resolution is a mixed blessing Very little is to be gained from it unless information is sought about the intracellular locus of incorporation The very fact that tritium labeled thymidine is confined to the nucleus and that its beta particles have a very short range, limits the maximum grain count over an average sized marrow cell to about 50 to 70 grains In the case of radioiron grain counts of several hundred are possible and indeed are relatively routine in our hands These high grain counts without intracellular radiation damage allow cells to be followed through many more divisions Technically, the lower resolution requires only that cell smears be dilute to prevent overlap, and that a good quantitative grain counting technic be developed to prevent introduction of errors due to cell size We have previously described such a method [1] Finally, it should be pointed out that if one wishes to study the life of a red cell from its early beginnings to the final non nucleated form a label must be chosen that will be part of the ultimate cell for this purpose iron labeling of hemoglobin is ideal

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Blood Uptake of Fe^{59} in Studies of Red Cell Production

By L F Lamerton, E H Belcher* and E B Harriss

In the application of Fe^{59} to the study of red cell production, one of the most convenient technics is the measurement of the rate of appearance in the circulating blood of radioactive iron that has been previously injected The blood uptake of intravenously administered Fe^{59} will be determined by the rate at which the various red cell precursors take up iron, their rates of maturation and release into the peripheral blood, the competition for iron from tissues other than the erythropoietic organs, and the extent of the utilization for erythropoiesis of Fe^{59} initially deposited in sites other than erythropoietic tissues and subsequently withdrawn or of Fe^{59} returned to the metabolic pool after red cell destruction

The blood uptake of Fe^{59} cannot, however, be considered a simple measure of red cell production, and in this paper its uses and limitations will be discussed, with examples taken from work with normal rats, irradiated rats, and rats treated with certain drugs

Materials and Methods

Male rats of the highly inbred August or Marshall strains were used, their behavior in respect to iron metabolism was found to be similar Radioactive iron, Fe^{59}, with a specific activity of 1 to 3 $\mu c/\mu g$ Fe, was injected as ferric chloride in 1 per cent sodium citrate solution

For the measurement of blood uptake of Fe^{59}, rats weighing from 120 to 150 Gm were given either 0 2 μc Fe^{59}, when blood samples of 0 5 ml were to be taken by cardiac puncture, or 1 μc Fe^{59}, when blood samples of 0 1 ml were to be taken from the cut tail Since it was found that the removal of relatively large blood samples from the heart caused appreciable stimulation of erythropoiesis, this procedure was abandoned in later experiments in favor of tail vein sampling Blood samples were assayed for Fe^{59} in a well type scintillation counter The results given are the means of groups of five animals

For autoradiographic studies, rats of 200 to 220 Gm were given 20 μc Fe^{59}, and smears were made of femoral marrow, the hind limbs being freed of circulating blood as completely as possible by perfusion with saline via the abdominal aorta After photographic processing the smears were stained for microscopic study, but, as no specific reticulocyte stain was used, immature erythrocytes were distinguished from mature erythrocytes on the basis of their polychromasia alone The numbers of red cell precursors present in femoral bone marrow were estimated by making differential counts on stained marrow smears and by deter-

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mining the total number of cells present by enumeration of a cell suspension in a hemocytometer

Whole body irradiations were given by exposure to x rays of 230 kvp, 1 5 mm Cu half value layer, at a dosage rate of 30 r/min Fuller details of the experimental procedures will be found in previous publications [1 2 6 7]

RESULTS

Withdrawal of Fe^{59} From Stores

Any analysis of the curve of blood uptake of Fe^{59} is difficult unless one can determine the proportion of the Fe^{59} appearing in the blood due to red cell precursors labeled directly by the injected Fe^{59}, and the proportion due to cells labeled with Fe^{59} originally deposited in storage sites and later withdrawn for erythropoiesis An attempt has been made to reduce such withdrawal by giving animals daily injections of an iron-dextran complex (Imferon) at a dose level of 5 mg Fe/100 Gm body weight Such daily injections of Imferon maintain the serum iron at about 10 times the normal level [8]

In figure 1a is shown the effect of Imferon injections starting 24 hours after intravenous administration of Fe^{59} on the blood uptake of Fe^{59} in normal Marshall rats, the blood samples being taken from the tail vein The curves give some indication of a lower final uptake in animals given Imferon but the difference is not sufficient for a firm conclusion to be reached When, however, blood up-

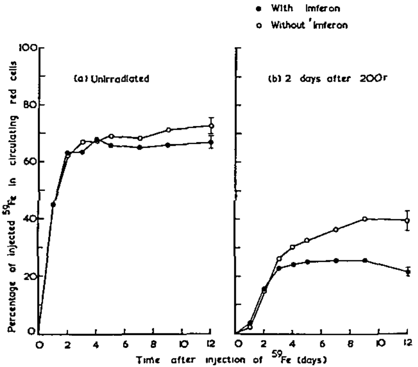

FIG 1—Effect of daily Imferon injections on blood uptake of intravenously injected Fe^{59} (Marshall rats)

takes are measured over the period of recovery from damage to the erythropoietic system, the effect may be very considerable, as illustrated in figure 1b, where the Fe^{59} injection was given two days after a dose of whole body radiation of 200 r.

From the curve of figure 1a relating to the Imferon injected animals it can be seen that more than half the Fe^{59} finally appearing in the blood is released within the first 24 hours after injection, and that there is very little increase after 48 hours. This indicates that all the iron incorporation in the red cell precursors of the normal rat takes place within 48 hours of emergence of the cell into the circulation and that the greater part of the incorporation occurs within the 24-hour period before emergence. The final level of Fe^{59} in the blood is about 65 per cent of the injected dose. This includes about 3 per cent of the injected dose directly incorporated into the reticulocytes of the blood during the time that the plasma level of Fe^{59} is high. In August rats, the final level of Fe^{59} in the blood is about 55 per cent of the injected dose.

Autoradiographic Studies of Uptake of Fe^{59} by the Red Cell Precursors

Autoradiographic studies of the rate of uptake of Fe^{59} into the red cell precursors of the bone marrow have previously been reported.[6] The conclusion from these studies is that, in the young growing rat, the cells in the stages from early normoblast to polychromatic erythrocyte in the marrow take up Fe^{59} at approximately the same rate. There is some uptake in the pronormoblasts but at a rather lower rate. A summary of the results obtained from smears made at two hours after Fe^{59} injection is shown in table 1. The polychromatic erythrocytes of the bone marrow account for about one third of the total Fe^{59} content. The remainder is found mainly in the intermediate and late normoblasts. The Fe^{59} content of the early normoblasts and pronormoblasts accounts for only about 5 per cent of the total. The autoradiographic studies show that the time from the early normoblast stage to emergence into the circulating blood is about 48 hours, consistent with the conclusion from the blood uptake data. It is important to recognize that the rates of maturation of the red cell precursors and the relative rates of iron uptake are very species dependent. The rat appears to have a much faster rate of maturation of red cell precursors and a relatively higher rate of iron uptake in the later forms than does man or the rabbit.[11] This species effect has always to be considered when comparing the results of different workers.

TABLE 1.—*Results Obtained From Smears Made at 2 Hours After Fe^{59} Injection*

Stage	Labeled cells per 100	Mean grains per labeled cell	Percentage of cells present	Fe^{59} content of stage (arbitrary units)	Fe^{59} content as percentage of total	
Pronormoblast	30	26	1	8	0.3	38.7
Early normoblast	90	34	5	153	5.2	
Intermediate normoblast	98	36	28	987	33.2	
Late normoblast	93	30	31	865	29.1	61.3
Polychromatic erythrocyte	91	30	35	955	32.2	

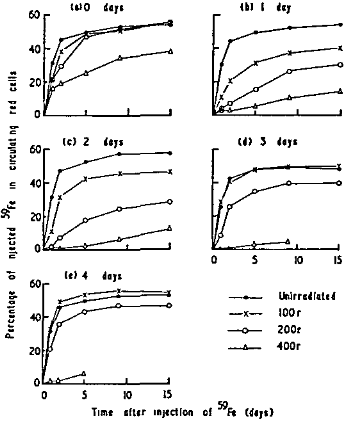

FIG. 2.—Effect of whole body irradiation on blood uptake of subcutaneously injected Fe^{59} (Marshall rats). Fe^{59} injected at various times after irradiation.

Blood Uptake After Whole Body Irradiation

In studying the effect on the erythropoietic system of a perturbation such as that produced by radiations, it is necessary to know whether there is any change in the rate of incorporation of Fe^{59} into the various red cell precursors. It has been shown by various workers[4, 9, 11] that the incorporation of iron into red cells is not appreciably affected by radiation doses as high as 1000 r.

Whole body irradiation given prior to the injection of Fe^{59} affects the rate at which labeled cells are released into the circulation. In figure 2 are shown blood uptake curves in Marshall rats subcutaneously injected with Fe^{59} at various times after exposure to whole body irradiation of 100, 200, or 400 r. In this experiment no Imferon injections were given and 0.5 ml. samples of blood were taken by cardiac puncture. In figure 3, a similar set of data is shown for August rats, but in this experiment only one dose level, 200 r, was used. The rats were given daily Imferon injections starting eight hours after intravenous administration of the Fe^{59}, and blood samples of 0.1 ml. were taken from the tail.

It can be seen from figure 2a that when the Fe^{59} is given immediately following irradiation with doses of 100 r or 200 r, the final level of Fe^{59} reached in blood is the same as that of the controls, but it takes a longer time to reach its final level. In the corresponding curve with the Imferon injected animals (fig. 3b),

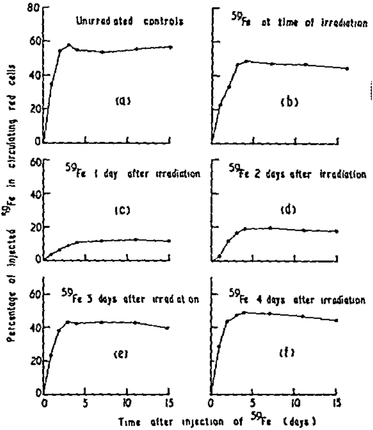

FIG 3 —Effect of whole body irradiation (200 r) on blood uptake of intravenously injected Fe^{59} (August rats) Fe^{59} injected at various times after irradiation Daily Imferon injections starting 8 hours after Fe^{59} injection

the final level is slightly below that for the controls, but the delay in emergence is very evident The simplest explanation of these results is that with doses of 200 r and less, there is little destruction by radiation of the iron incorporating cells, at least in the intermediate and late normoblast and polychromatic erythrocyte stages, but there is a delay of release or maturation of these forms When *the Fe^{59} is given one or two days after the irradiation with a dose of 100 r, and one, two, or three days after 200 r, there is not only a delay in emergence of Fe^{59}* but also a marked reduction in the final level reached in the blood

The curves for animals given 400 r show a profound effect, suggesting that a large proportion of the iron incorporating precursors do not emerge into the circulation The results for 400 r are incomplete because a number of animals died from hemorrhage following the cardiac puncture

From the results of the experiments with the Imferon injected animals, one can show that following irradiation the relationship between uptake of Fe^{59} by the red cell precursors and time before emergence is not the same as in the control animals With a dose of 200 r this effect is particularly marked in the cohort of cells released into the blood during the interval of two to three days after irradiation When the Fe^{59} is given at the same time as irradiation, this cohort of cells will emerge between two and three days after the injection, from figure 3b it can be found that their Fe^{59} content is about 12 per cent of the injected dose When the Fe^{59} is given two days after irradiation, this cohort of cells will emerge during the first day after the injection, from figure 3d it can be seen that their Fe^{59} content is only 2 per cent of the injected dose A similar result is found if one considers cohorts of cells released at other times following the irradiation It must be concluded that the cells in the marrow at the time of irradiation tend to lose their capacity for Fe^{59} incorporation as they approach the time of emergence This is contrary to the observations in the normal animals

A possible explanation of this effect is that radiation causes a delay in release, though not in maturation, of the later forms of red cell precursors in the bone marrow The later forms would continue to take up iron and could acquire their full complement of hemoglobin earlier relative to the time of emergence than in the case of normal animals If this were so, the cells emerging, for instance, between two and three days after 200 r whole body radiation might have very little capacity for Fe^{59} uptake

Cell Counts in Irradiated Animals

To show whether there is, in fact, a hold-up of the later forms in the bone marrow following whole body irradiation, absolute and differential counts were made of red cell precursors in femoral bone marrow at times up to 14 days after 200 r whole body radiation This work has been reported elsewhere,[7] and the results are summarized in figure 4 The precursors up to the late normoblast stage show a rapid fall in numbers soon after irradiation, consistent with the

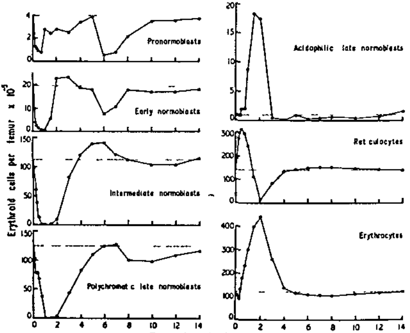

FIG 4—Variation with time in population of erythroid cells in femoral bone marrow after 200 r whole body irradiation (August rats)

radiation having its major effect on the pronormoblast or even earlier stages. There is a second fall in the number of pronormoblasts, which is discussed later. The late forms, however, show a very different pattern of response. One striking change in the bone marrow population is the appearance of abnormally large numbers of acidophilic late normoblasts two days after irradiation. These cells are comparatively rare in the normal rat, but two days after irradiation, they represent no less than 80 per cent of the nucleated erythroid cells. Within 24 hours of irradiation the number of polychromatic erythrocytes shows a great increase, they then decrease, while the number of erythrocytes continues to rise. The observed increase in erythrocyte population might be a result in part of incomplete exsanguination of the animal before the bone marrow samples were taken, and might also be due in part to hemorrhage from the circulating blood into the marrow parenchyma. However, these factors would not account for the large number of immature erythrocytes seen. Their presence supports the conclusion that there is a delay in the emergence of cells after irradiation, and that they emerge at a more mature stage than in the normal animal.

The delay in emergence of the mature cells could be explained if one assumes that the "growth pressure" of the earlier precursors is a major factor in determining the release of the later forms from the bone marrow. Since the effect of radiation is to deplete the population of earlier precursors, the normal rate of release might occur only when regeneration has re-established a full population of early precursors.

One must conclude, therefore, that following damage to the bone marrow the rate of appearance of previously administered Fe^{59} into the blood cannot necessarily be taken as a measure of the rate of emergence of new red cells into the circulation. For the same reasons, the reticulocyte count is also not necessarily a measure of the rate of emergence of new red cells. However, in spite of this limitation, or even because of it, the peripheral blood uptake of Fe^{59} can provide a very sensitive indication of the effect of radiation or of other damaging agents, conveying essentially the same type of information as the reticulocyte count, but in a more quantitative manner. A convenient index is the Fe^{59} uptake of the blood 24 hours after injection. A demonstration of the way in which the 24-hour uptake of Fe^{59} changes with time after various doses of whole body radiation is shown in figure 5. Results are given for the two strains of rats we have used, and the agreement between them is reasonably good.

For the purpose of quantitative comparison of the effects on the erythropoietic tissue of different radiation conditions or of other agents, a better measure than the 24 hour blood uptake of Fe^{59} is probably the final level in the blood when daily Imferon injections are given to suppress withdrawal of Fe^{59} from stores. This should represent the fraction of the injected Fe^{59} which is taken up initially by the erythropoietic tissues. However, the final level will not be proportional to the iron incorporating capacity of the erythropoietic tissues, unless the effect of any changes in plasma clearance rate of iron and plasma iron concentration compensate each other. The available data do not allow one to be certain on this point. The values of the final level of blood uptake of Fe^{59} from Imferon injected animals, at various times following a dose of 200 r, are given in figure 6.

An interesting feature of this curve is the second fall occurring between 7 and

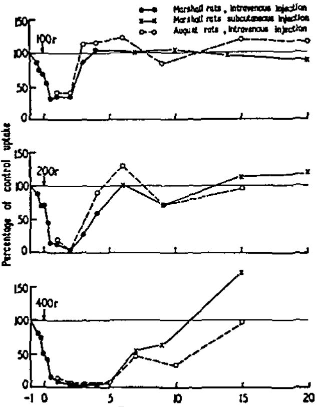

FIG 5 —Effect of whole body irradiation on 24 hour uptake of Fe^{59} in blood Fe^{59} injections given at various times relative to irradiation

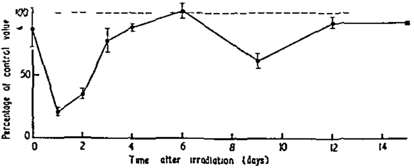

FIG 6 —Maximum uptake in the blood of Fe^{59} injected at various times after 200 r whole body radiation (August rats) Daily Imferon injections starting 8 hours after Fe^{59} injection

12 days after irradiation A similar fall is also evident in the counts of pronormoblasts and early normoblasts in figure 4. It is not yet clear whether this is a result of a particular type of damage produced by ionizing radiation, or whether it is a manifestation of a more general aspect of the recovery process Comparative studies with various cytotoxic drugs may throw light on this question [5]

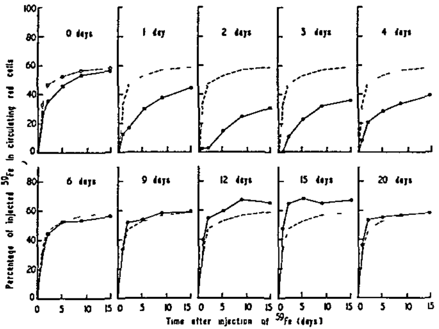

FIG 7 —Effect of administration of Chlorambucil (12.5 mg /Kg) on blood uptake of subcutaneously injected Fe^{59} (Marshall rats) Fe^{59} injected at various times after administration of drug Control values shown as dotted curve

blood uptake curve, apart from change in absolute values of uptake, could indicate changes in rates of cell proliferation or of maturation in the iron incorporating stages of the precursors

Our only comprehensive series of data on blood uptake of Fe^{59} during recovery of the erythropoietic system has been obtained not with radiation, but after treatment of the rat with the nitrogen mustard, Chlorambucil In this experiment, Fe^{59} was given subcutaneously, 0 5 ml samples of blood were taken by cardiac puncture, and Imferon was not given The data (fig 7) indicate the type of results that can be obtained The blood uptake curves show a progressive change in shape an uptake slower than normal in the first 4 days after the administration of the drug, normal between 6 and 9 days, and between 15 and 20 days there was evidence that the final uptake was reached more quickly than in the control rats Also at 15 days the final uptake is somewhat greater than in the controls During the first 4 days after administration of the drug, the slower rate of uptake of Fe^{59} in the blood can be ascribed, at least in part, to the slower release of the later forms, as already described for irradiated animals, although withdrawal from stores and reutilization of Fe^{59} are likely also to have affected the shape of the curves

The more rapid uptake at 15 and 20 days (and possibly at 12 days) suggests an increased rate of division or faster rate of maturation of the iron incorporating red cell precursors during the period of recovery This is consistent with a direct action of the erythropoietic-stimulating factors on the later stages of iron incorporation, but there may be other explanations, and a more definite conclusion must await further experimental data

SUMMARY

By measurement of the rate of appearance in the circulating blood of previously injected radioactive iron (Fe^{59}), a study can be made of certain aspects of red cell production and of changes in the population of red cell precursors consequent upon damage to erythropoietic tissue The limitations and applications of this technic are discussed, with examples from studies with normal rats irradiated rats, and rats treated with a nitrogen mustard

For quantitative work, it is important to suppress as far as possible the withdrawal for erythropoiesis of Fe^{59} originally deposited in storage sites of the body, this can be achieved by maintaining the nonradioactive iron content of the serum at a high level

It is shown that following damage to the erythropoietic tissues, the blood uptake of Fe^{59} does not necessarily provide a direct measure of the rate of red cell production The most useful quantitative criterion is probably the final level of Fe^{59} reached in the blood when withdrawal of Fe^{59} from stores has been suppressed

The blood uptake of Fe^{59} gives only an integrated measure of erythropoietic activity and in experimental animals there may be important differences in the erythropoietic activity of different erythropoietic sites

Finally, the changing pattern of blood uptake of Fe^{59} in various stages of recovery of the erythropoietic tissue from damage is discussed

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Control of the Proliferative Steady State

By Allan J. Erslev

THE TISSUE REQUIREMENTS for oxygen in the mammalian organism are met by the oxygen brought in from the atmosphere through the integrated action of lungs, heart, vessels, and bone marrow. Under physiologic conditions, the supply of oxygen is in perfect balance with the demand, indicating that an efficient feedback mechanism must be operating between the tissues and each of the organs involved in the oxygen transfer. These feedback mechanisms are obviously mediated in one direction by oxygen flowing to the tissues, but how they are mediated in the opposite direction is still highly speculative. It has been demonstrated[16] that the lungs are influenced by tissue hypoxia through local and generalized changes in pH, but the exact impulses and mechanisms that regulate vessels and heart are not known.[8] With regard to the bone marrow, however, *recent studies have begun to make clear the nature of the impulses that control* the marrow's contribution to the oxygen transport.

The only known function of the erythropoietic tissue is to produce hemoglobin containing red blood cells, and the main physiologic function of these cells is to transport oxygen to the tissues. Consequently, it has generally been accepted that the control of the proliferative rate of the erythropoietic tissue depends mainly if not exclusively on some effects of oxygen on tissue metabolism. Such a control could be accomplished by a simple feedback mechanism if the proliferative activity of nucleated red cells is accelerated when the cells are deprived of oxygen, and suppressed when the cells are exposed to high concentrations of oxygen. However, Thomas has reported[15] that the 10-hour synthesis of hemin in a suspension of rabbit bone marrow does not vary in a reciprocal manner with oxygen tension. These results actually do not rule out a reciprocal relationship between proliferative activity of the erythropoietic tissue and oxygen tension, since it is unlikely that an alteration in the rate of proliferation would result in substantial changes in the synthesis of hemin in this short period of time. Rosin and Rachmilewitz,[12] as well as Magnussen,[10] have reported an inhibiting rather than a stimulating effect of low oxygen tension on bone marrow cultures, but these tissue cultures are hard to evaluate quantitatively. In our laboratory recent studies carried out in collaboration with James Hughes have shown that a bone marrow suspension under near physiologic conditions will respond to a low oxygen tension, both with a decrease in the hemoglobin synthesis and a decrease in proliferative activity. Normal rabbit bone marrow, dispersed by means of stainless steel screens, as recommended by Thomas,[15] was suspended in normal serum and divided into aliquots. Radioactive iron preincubated with normal serum was added, and the mixture was incubated in a Dubnoff incubator at a pH of 7.4, at a temperature of 40C., and under an atmosphere of 5 per cent O_2 and 5 per cent CO_2 (corresponding to the tensions in venous blood), or 13 per cent O_2 and 5 per cent CO_2 (corresponding to arterial blood). Samples were re-

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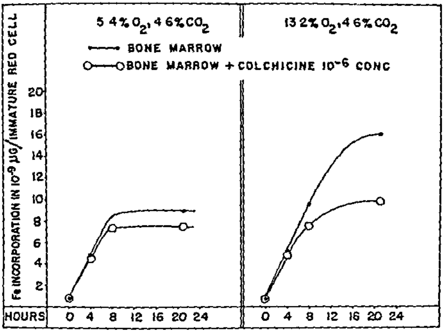

FIG 1 —Solid dots represent iron uptake by nucleated red cells and reticulocytes in a bone marrow suspension incubated for 21 hours at 40°C at a pH of 7 4 and in an atmosphere of either 5 per cent O_2–5 per cent CO_2, or 13 per cent O_2–5 per cent CO_2 Open circles represent iron uptake by the same bone marrow suspension under the same conditions except for the addition of 1 µg of colchicine per ml of suspension

moved after 4, 8, and 21 hours Metabolism was arrested by means of sodium cyanate the cells washed five times, and the cellular uptake of radioactive iron measured in a scintillation counter In order to evaluate the proliferative rate, control samples were treated in an identical manner, except for the addition to the marrow suspension of 1 µg of colchicine/ml Figure 1 charts the results of one such experiment The rate of hemoglobin synthesis (4 hour value) increased slightly with increasing oxygen tension and was not influenced by the presence of colchicine The rate of mitotic activity estimated from the relative difference between the 21 hour samples with and without colchicine, also increased as the oxygen tension increased

These results do not rule out the possibility that the rate of stem cell differentiation is accelerated by a low oxygen tension, since it is questionable if differentiation actually takes place in an in vitro system However, the recent demonstration of an erythropoietic factor in the circulating blood of anemic animals has made it quite unlikely that the oxygen tension in the bone marrow is responsible for the physiologic regulation of the rate of red cell production The existence of a humoral factor with erythropoietic properties has led to a new working hypothesis, which proposes that tissue hypoxia causes the production or release of an erythropoietic factor and that the level of this factor in serum controls the proliferative activity of the erythropoietic tissue

There is much experimental support for this hypothesis, although it is far from proved [13] Serum from normal animals is inactive or has only slight activity, while serum from anemic or hypoxic animals is very active, an activity that appears to be roughly proportional to the degree of anemic or hypoxic anoxia Furthermore serum becomes erythropoietically active a few hours after the onset of anoxia, and the activity disappears two to four hours after the discontinuance of the anoxic state[3]

Consequently, the erythropoietic steady state may depend on a feedback mechanism that operates between the erythropoietic tissue of the bone marrow and one or many oxygen sensitive tissues and that is mediated by oxygen in one direction and by the erythropoietic factor in the opposite direction So far no one organ or tissue can be singled out as being solely responsible for the elaboration of erythropoietic factor Even the kidney which has received a great deal of attention recently, does not meet the necessary requirements Mirand and Prentice[11] have been unable to confirm studies reported by Jacobson and co workers[7] that serum from hypoxic nephrectomized rats is devoid of erythropoietic activity Studies in our laboratory have also demonstrated that nephrectomized rabbits are capable of elaborating small amounts of erythropoietic factor[4] More significant is the fact that erythropoietic tissue in rabbits continues to be active long after the complete removal of both kidneys Radioactive iron continues to be removed from serum and incorporated into new red cells and as many as nine days after nephrectomy young reticulated red cells were found in increased numbers in the blood stream[5] Furthermore, the rate of radioactive iron turnover in nephrectomized rabbits can be shown to respond to anemic anoxia and to hyperoxia in a physiologic manner, although quantitatively the response falls far short of normal (table 1)

These considerations would tend to rule out the kidneys as the only source of erythropoietic factor Since animals without spleen, thymus or endocrine organs and animals treated with x radiation or nitrogen mustard are capable of elaborating erythropoietic factor it is conceivable that there may not be one organ or one tissue system solely responsible for the production of erythropoietic factor It may be that a great variety of tissues dependent on aerobic metabolism are capable of releasing erythropoietically active substances when lack of oxygen forces them to utilize anaerobic metabolic pathways

The manner with which the erythropoietic factor after having been released

TABLE 1 —*Serum Iron Turnover After 48 Hours of Anemic Anoxia or Hyperoxia*

	Hematocrit		NPN	Serum iron turnover mg. day/100 ml.	RBC utilization 70 hours%
	No	%	mg. %		
Normal rabbits	7	42	46	2 22 ± 0 54	38 ± 10
Normal rabbits Bled 20 ml /Kg	4	27	47	5 15 ± 1 45	97 ± 14
Normal rabbits Exposed to 68% O_2	5	43	40	1 69 ± 0 25	39 ± 4
Nephrectomized rabbits Bled 20 ml /Kg	8	23	119	3 71 ± 1 03	42 ± 21
Nephrectomized rabbits Exposed to 68% O_2	8	38	125	2 42 ± 0 55	56 ± 13

into the circulating blood regulates the proliferative activity of the erythropoietic tissue has been studied for some time in our laboratory. It seems almost certain that this regulation must be accomplished either by a stimulation of the rate of mitotic division of the nucleated red cells or by a stimulation of the rate with which multipotential stem cells are differentiated into pronormoblasts. However, it has been demonstrated that the rate of mitotic division of nucleated red cells is the same in normal animals and in animals in which the erythropoietic tissue was stimulated by means of anemic anoxia or suppressed by means of exposure to high oxygen tension.[6] Consequently, the erythropoietic factor must act by accelerating the differentiation of stem cells into pronormoblasts and is not required for subsequent multiplication of the differentiated nucleated red blood cells. This explains why the ratio between early and late nucleated red cells usually is the same in normal bone marrow and in bone marrow stimulated by tissue anoxia or suppressed by tissue hyperoxia.[2, 9, 14] Furthermore, Alpen and Cranmore[1] have shown that the intermitotic interval of nucleated red cells in normal and anemic dogs is the same. Finally, there is the well known observation that it takes several days from the onset of a hypoxic stimulus or a hyperoxic suppression until a change in the peripheral reticulocyte count can be observed. If the rate of mitotic activity actually was regulated by a circulating erythropoietic factor, a much more rapid change in the reticulocyte count could be anticipated. Figure 2 shows that colchicine, a mitotic poison, will suppress the output of reticulocytes in rabbits almost immediately, while the exposure to

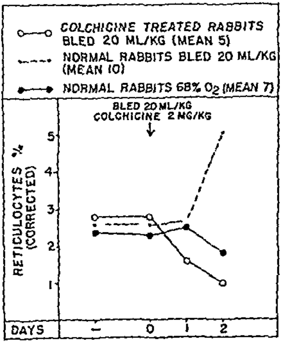

FIG. 2.—Mean reticulocyte counts expressed in per cent of baseline red cell count of rabbits bled 20 ml./kg. (dotted line), bled 20 ml./kg. and given colchicine 2 mg./kg. (lines with open circles), or kept in an atmosphere with 68 per cent oxygen (lines with solid circles).

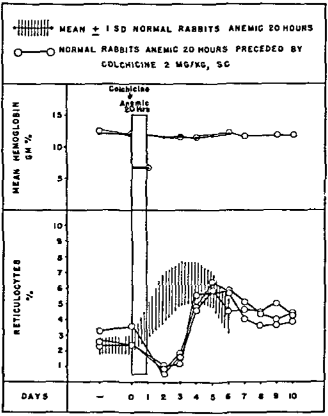

FIG 3 —The shaded area represents the mean reticulocyte response ±1 standard deviation of 10 normal rabbits kept anemic for 20 hours, and the solid lines the mean hemoglobin concentration Lines with open circles represent the mean hemoglobin and individual reticulocyte response of 3 rabbits given colchicine 2 mg /Kg , s c 2 hours before the onset of a 20 hour period of anemia (From an article to be published in *Blood* the Journal of Hematology Reproduced with the Editor s permission)

high atmospheric oxygen first influences the reticulocyte count after a delay of 24 to 48 hours

The biologic nature of this erythropoietic factor–dependent process of differentiation is unknown, but it does not appear to involve a mitotic division since it is uninfluenced by colchicine Figure 3 shows that the administration of colchicine to rabbits just before a 20-hour period of anemic anoxia will not abolish the normal reticulocyte response but merely postpone it This must mean that stem cells were differentiated into pronormoblasts during the 20 hour period of anemic anoxia, despite the colchicine-induced block of mitotic activity As soon as the colchicine effect was terminated, these new pronormoblasts proceeded to divide and mature in a normal manner, resulting in a late but otherwise characteristic reticulocyte peak

In conclusion, it appears that the homeostatic mechanism that balances red cell production with the need for oxygen in the tissues operates as a feedback mechanism, mediated from the bone marrow to the tissues by hemoglobin bound oxygen, and from the tissues to the bone marrow by a humoral erythropoietic factor The site and action of oxygen in the tissues leading to the elaboration of erythropoietic factor are still unknown However, the mode of action of erythropoietic factor in the bone marrow is better understood since it appears to control the rate of differentiation of undifferentiated stem cells to early nucleated red cells

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Observations on the Kinetics of Red Cell Proliferation

By FREDERICK STOHLMAN, JR.

CHANGES in the rate of red cell production occur promptly after alterations in atmospheric pO_2 or circulating red cell mass. It has been established that increases in red cell production in response to hypoxia are mediated through a hormone, erythropoietine,[1] and it has been suggested that a second regulant in addition to erythropoietine is involved in situations where the circulating red cell mass is altered.[2] The mechanism by which these regulants affect the rate of cellular proliferation is not established. It was considered that the rate of cellular production could be influenced by (a) alterations in the number of dividing progenitor cells, (b) changes in intermitotic interval, (c) changes in the death rate of cells during the production process, i.e., change in the "abortion" rate or (d) a combination of two or more of the above mechanisms. One also might consider the possibility of cells becoming dormant for variable periods, as has been suggested for leukocytes[3] and spermatogonia.[4] In effect, however, this is a specialized case of change in intermitotic interval. Observations bearing on which of these mechanisms is responsible for alterations in red cell production following appropriate stimuli are the subject of this report.

MATERIALS AND METHODS

Relative rates of red cell formation in 140 to 160 Gm. Sprague Dawley rats were estimated by red cell Fe^{59} uptake and reticulocyte counts. Two Fe^{59} technics were used. In the first, Fe^{59} labeled plasma was given intravenously and three hours later, when 90 to 95 per cent of the radioiron had been removed from the plasma, 0.1 ml. of Imferon* was given intramuscularly. Imferon was given daily thereafter. In other experiments Fe^{59} labeled plasma was given and followed in three hours by Imferon. Then, 30 to 60 minutes after the administration of Imferon, the experimental procedure was performed. Blood volumes were determined in experimental animals with the dilution technic, injecting 0.3 cc. of Cr^{51} tagged cells 20 minutes prior to exsanguination. Radioactivity was measured in a well type scintillation counter, using a single channel spectrometer to discriminate between Fe^{59} and Cr^{51}. Plasma containing erythropoietine was obtained from Sprague Dawley rats exposed to 23,000 feet of simulated altitude for 18 hours.[5]

RESULTS

(a) *Fe^{59} Incorporation in the bled animal.* As might be expected, rats that were bled from 1 to 2 per cent of the body weight three days prior to the administration of Fe^{59} had a substantial increase in the percentage of injected iron appearing in the peripheral erythrocytes. Maximum Fe^{59} incorporation in the bled animals was achieved 48 hours after injection, while in normal rats it was ob-

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From The National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Md.

* Imferon, an iron-dextran complex containing 50 mg. of iron per ml.

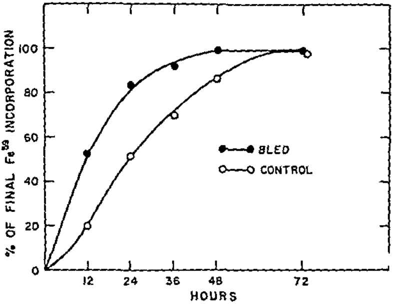

FIG 1 —The fraction of final iron incorporation appearing at intervals after injection of Fe^{59} Experimental animals were bled 2 per cent of the body weight three days prior to injection of Fe^{59} Each point represents mean value for six animals

served between 48 and 60 hours When the fraction of the total incorporated iron appearing in the peripheral blood was calculated for various time intervals, it was evident that the bled animals had a much higher proportion of iron appearing in the first 24 hours than did the normals (fig 1)

(b) *Effect of bleeding and transfusion when performed after the administration of Fe^{59}* In a series of experiments, Fe^{59} was administered to a group of normal animals Three hours thereafter Imferon was given, followed by either bleeding or transfusion Measurement of incorporation of Fe^{59} together with blood volumes were carried out at intervals thereafter The results of three such experiments are presented in figure 2 During the first 12 hours, Fe^{59} incorporation in both groups was identical in all three experiments In part, this was accounted for by an uptake of about 6 to 8 per cent by peripheral reticulocytes At the end of 24 and 36 hours, however, the bled group had a higher incorporation than did the transfused group The final Fe^{59} incorporation of bled groups in all instances was greater than for transfused animals The maximum values in both groups were observed after 48 hours

(c) *Effect of erythropoietine on cellular proliferation* In two experiments, Fe^{59}-labeled plasma was given to two groups of rats that had been hypertransfused 72 hours previously Three hours after the administration of Fe^{59}, Imferon was given, and 30 to 60 minutes later, normal rat plasma was given to one group and erythropoietine containing plasma obtained from animals exposed to altitude was given to the other group The results are presented in figure 3 Both experiments gave similar results The 12 hour Fe^{59} incorporation of the recipients of normal and altitude plasma was identical The recipients of erythropoietine had a higher Fe^{59} incorporation at 24 hours, by 36 hours the difference was substantial The final Fe^{59} incorporation of erythropoietine treated animals, 23 per cent

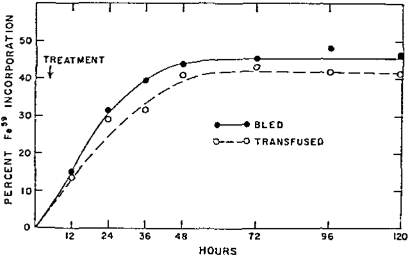

FIG 2 —The curves represent the average iron incorporation as per cent of injected dose, in three experiments Each experimental point represents the mean value for 18 rats Treatment was carried out three hours after injection of Fe^{59}

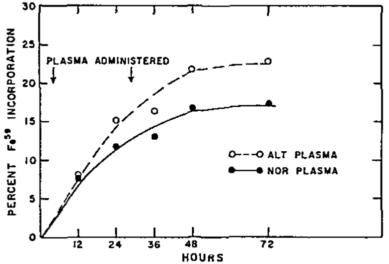

FIG 3 —The curves represent average iron incorporation, as per cent of injected dose in two experiments Animals were hypertransfused three days prior to injection of iron Each point represents average value for 12 animals

of the injected dose, was significantly greater than the 16 per cent incorporation of the animals receiving normal plasma The p values in both experiments were less than 01

DISCUSSION

The administration of a ' flooding" dose of nonradioactive iron at an interval after the injection of Fe^{59} limits the period during which Fe^{59} is available for uptake by red cell precursors Theoretically, this maneuver should lead to a more sharply defined peripheral Fe^{59} appearance (so-called incorporation or uptake) curve The interval between injection of radioiron and maximum peripheral value provides an estimate of the emergence time, i e , the time for the most immature labeled cell to divide, mature, and enter the peripheral blood It should be noted that in the absence of a flooding dose of nonradioactive iron, the total iron incorporation and the interval over which it appears is greater[5] The additional uptake occurring under such circumstances is thought to be due to reutilization of Fe^{59} following death of cells within the marrow, together with re-entry of Fe^{59} from tissue sources

An analysis of the proportion of the total incorporated Fe^{59} appearing in the periphery during each time interval after injection should give a rough estimate of the distribution of the various stages of erythrocytic precursors Thus, any change in the proportion of Fe^{59} appearing in a given time interval would reflect either a change in the proportion of cells in various stages or a change in generation time

When an experimental procedure is preceded by the administration of Fe^{59} and a flooding dose of nonradioactive iron, one can determine the effect of that treatment on a specific cell population, the cell population being confined to those labeled by Fe^{59} The degree of labeling of precursors may vary with species In the case of the rat, it has been demonstrated autoradiographically[6] that there is equal labeling in all red cell precursors from acidophilic normoblasts through late orthochromatic normoblasts Pronormoblasts take up less radioiron per cell, and the earlier stages none at all It has been reported in dogs,[7] rabbits,[8] and man[9] that the pronormoblasts take up more iron than do later stages In all species, however, it has been observed that the early progenitor cells are not labeled by radioiron In the type of experiment described here, changes in the peripheral Fe^{59} appearance curve reflect only an alteration in the proliferation of cells previously labeled An influx of unlabeled early progenitor cells as a source of increased production would not alter the curve Thus, observed changes in the curve would be attributed to changes in intermitotic interval, maturation time, or the death rate of cells within the marrow

Two possible schemes of red cell generation are represented in figure 4 In the first, division occurs at each stage of maturation, in the second, maturation is not necessarily preceded by division In either case, death of cells or dormancy could occur in the course of proliferation The present studies do not permit a conclusion about whether all cells of a given cell type divide once or many times before maturing, or whether maturation may occur without division Harriss[6] and Alpen[7] observed a decrease in the mean grain counts of the earliest labeled (Fe^{59}) red cell precursors, indicating division without maturation, i e , pronormoblast divides into pronormoblasts The rate of fall of the mean Fe^{59} grain count in the intermediate and late normoblasts, reported by Harriss,[6] suggests that normally some cells mature without division

It is evident from figure 1 that the *fraction of the total radioiron appearing in* the first 24 hours is substantially greater in bled than in normal animals Moreover, the observed emergence time (time for the most immature labeled cell to reach the peripheral blood) in bled rats was shorter than that noted in control animals From this it may be concluded that there was a decrease in intermitotic

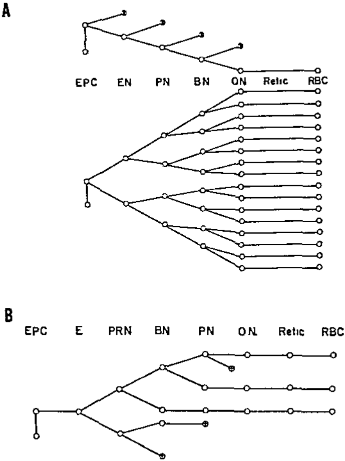

FIG 4 —Schematic representation of red cell proliferation EPC—early progenitor cell, E—erythroblast, PRN—pronormoblast, BN—Basophilic normoblast PN—polychromatophilic normoblast, ON—orthochromatic normoblast, Retic reticulocyte RBC red blood cell 4A depicts extreme perturbations of a system with fixed maturation times but where cell death occurs, 4B the normal in a system in which both are variable

interval (including dormancy) and/or maturation time A simple decrease in these parameters would lead to a temporary increase in output A sustained increase in output would depend upon an increased input, i e, division either from early precursor cells or from an increase in the number of divisions as cells differentiate, between pronormoblast and orthochromatic normoblast Teleologically one might suggest that this latter would be an ineffective mechanism since it would require initially a temporary decrease in the red cell output However, if there was a simultaneous decrease in death rate this might maintain the cellular output during the interval when the number of maturing cells was decreased *to permit an increased number of divisions If the number of divisions increased* then the intermitotic interval and maturation time would necessarily have to decrease, or an increase in emergence time would result Since shortening of total peaking time was observed, the decrease in intermitotic interval becomes sub*stantial Alpen[7] has presented data that ostensibly demonstrate no change in the* average number of divisions following bleeding His conclusion however is based on these assumptions that bleeding does not change the relative proportion of nucleated cells i e orthochromatic normoblast to pronormoblast or the intermitotic interval and that there is no death of cells in the marrow Indeed the evidence presented herein raises serious doubt as to the validity of these assumptions It should be noted that technics depending on labeling at the time of DNA synthesis would not detect the maturation of early cells without division except on rare occasions when a polyploid cell would result

The studies on rats given iron and subsequently undergoing transfusion or bleeding or receiving erythropoietine (figs 2 and 3) are compatible with the hypothesis that cell death does occur in the marrow In these animals where labeling had already occurred when treatment was instituted there was a greater total Fe^{59} incorporation in the erythropoietine treated group than in the recipients of normal plasma In three experiments a similar though less marked difference was noted between the bled and transfused animals. These results might be interpreted as due to either a decrease in the death rate of red cell precursors or a reutilization of radioiron Reutilization of Fe^{59} could occur either in chunks from cells dying in the marrow or as the result of an ingress of radioiron from tissues The latter seems unlikely in view of the dilution of Fe^{59} with the large flooding dose of nonradioactive iron which raised the serum iron tenfold[10] and substantially reduced the specific activity The other possibility that labeled cells die are phagocytosed and the label reutilized appears to be more feasible In favor of this explanation is the observation by Alpen[7] who has shown by autoradiography that Fe^{59} appears in reticulum cells of bone marrow 12 to 24 hours after administration of the Fe^{59} Moreover Bessis[1] has demonstrated the transfer of ferritin from these reticular cells to erythrocytic precursors The only argument against the explanation that erythropoietine reduces the death rate of red cell precursors is the inability to demonstrate such an effect in vitro[12] In either event however death of cells in the course of proliferation is implicated The death of red cell precursors during the process of generation is in concert with the view advanced by Brecher et al in regard to lymphocytes[13] One might speculate that an aberration of this mechanism leads to the marked intramarrow death of cells which has been reported in such diseases as Cooley s anemia and is referred to as ineffective erythropoiesis by Giblett[14] and Sturgeon[15]

The data do not permit any final conclusions as to the exact mechanisms of cell production they demonstrate that normally a significant number of erythrocytic precursors are destroyed in the marrow There is also a shortening of the emergence time following severe hemorrhage The data also raise serious doubt as to the validity of those data based on the assumption of equivalency within the generative compartment

Piecing together information obtained from the present series of experiments together with data presented from other sources one might speculate that normally red cell production proceeds as depicted in figure 4b At each cell stage there is a mean probability that division or maturation will occur For each stage the probability of division may differ and beyond the polychromatophilic normoblast division does not occur In addition there is a normal attrition of cells in the marrow Increased production occurs by virtue of a decrease in the death rate as well as an increase in the number of divisions There is a decrease in generation time due to shortening the intermitotic interval, and possibly of maturation time to compensate for an increase in the number of divisions

It should be noted in considering possible generation schemes that one is studying average cell populations and that division or maturation is not synchronous, nor are mitotic time intermitotic intervals, etc , constant There is a mean mitotic interval with a distribution that is probably normal so also for other variables On the surface variation in a number of parameters as undoubtedly occurs in cellular proliferation, appears to lead to an unstable situation However as McCulloch[16] has pointed out, in suitably constructed systems instability of individual units may lead to desired flexibility and rapid response to stimuli

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Discussion of Kinetics of Red Cell Proliferation

F Stohlman, Jr In the Suit formulation[1] for calculation of the total number of nucleated red cells in the marrow it is assumed that all of the cells which are labeled eventually reach the peripheral blood If ineffective erthropoiesis occurs, this formulation cannot be used It is also assumed that all of the labeled cells reaching the periphery are labeled within the first 24 hours of the administration of iron That this is not the case, however, has been shown by Lamerton et al[2] and Harriss,[3] who demonstrated a reduction in final total incorporation when Imferon was given 24 hours after Fe^{59} We have similar data

As I interpret Dr Alpen's model, normally the erythroblast divides into two cells, one of which matures and as a more mature cell goes through a doubling division (about 5 per cent of the erythroblasts mature without dividing), the other cell remains as an erythroblast. The latter cell presumably retains one-half the label, when it divides again, it provides another labeled cell and so on ad infinitum Do you determine the number of divisions prior to emergence by dividing the numbers of labeled nucleated cells at a given time by the number of labeled cells eventually appearing in the periphery? If there is a continuous generation of labeled cells, can this be valid?

The generation time of the erythroblast is determined by the fall in grain count Did you suggest that the fall in the grain count in bled animals is due to an influx of stem cells? Would you clarify that?

E L Alpen The rate of decrease in grain count is the same for bled and normal dogs The basis for my argument is that the rates of decline are indistinguishable The *percentage of labeled cells*, however, decreases more rapidly in the bled than the normal As Dr Bond emphasized, grain count can change only by cell division The percentage of labeled cells is influenced only by the influx of unlabeled cells If Suit's technic is wrong and there is appreciable cell death, it can be wrong by as much as 10 or 15 per cent It would not matter in this case if the error was as much as 30 per cent It still could not account for the sixfold increase in cell production during the time that we are measuring it

We establish what we think are the most likely number of divisions by determining the ratio between the grain count in the erythroblast at the start of the experiment, and that finally present in the mature red cell This is not necessarily the actual number of divisions occurring, but only a limit that we are able to put on it We can say that it is no less but it could be a good deal more than that We actually can follow a cell through about three divisions, and then it becomes to all intents and purposes negative, due to dilution of the label to a point where the radioactivity approaches background

F Stohlman, Jr Some of the injected iron initially will go to the tissues At a later date it will return to the plasma and thence to the bone marrow, where it will be utilized in hemoglobin synthesis Will not such reutilization affect your curves and calculations?

E L Alpen The question of reutilization is a very serious one However, it does not become a problem in autoradiography The iron disappearance curve has two components, one being very steep, with a half time of about 70 minutes It is during this period that the cells we see in autoradiographs are labeled The second component has a half time of several days, but the specific activity is about 01–0 1 per cent of the maximum Any cells labeled at this level of radioactivity would not appear as positively labeled However, the second component would affect a peripheral iron uptake curve This may account for the difference between my bled and control animals, because in the bled animals there was no second component Nearly 100 per cent of the dose is taken up in the first phase

A Erslev If I understood you correctly, you felt that mature red cells with very high grain counts must have originated directly from the pronormoblast without any further divisions However, reticulocytes are almost as avid for radioactive iron as pronormoblasts Therefore, at the time of labeling, the immature reticulocytes may have been as intensely labeled as the pronormoblast

E L Alpen *Do you refer to the rat?*

A Erslev No the rabbit

E L Alpen It is not true for the rabbit, and I certainly know it is not true for the dog or human Immediately after labeling if the average grain count of a reticulocyte is 10 then the average grain count of erythroblast would be 250 to 300

A Erslev Didn t Dr Lamerton show some data to the contrary in the rat?

E L Alpen The rat is a very unusual species in that hemoglobinization does not begin until late in development of the red cell This is not true in man dog or rabbit In these species hemoglobinization starts at the time the cell becomes an erythroid precursor and there will be a six to eightfold difference between the intensity of label of erythroblast and reticulocyte

C P Leblond Are all these cells able to take up radioactive iron? Where does it stop? Immediately after mitosis?

E L Alpen In the dog iron is incorporated by all erythroid cells from the earliest erythroblast to the most mature reticulocyte

C P Leblond Is it not possible to explain the difference in the grain count by the fact that different cells would begin taking up iron at different times or that the rate of incorporation of various types would be different?

E L Alpen Nearly all cells will be labeled within two hours of injection of iron The relationship between the grain count of the cell and its state of maturation *is a simple one It isn t because it is an* erythroblast that it makes hemoglobin twice as fast It is related only to the cytoplasmic volume of the cell If we assume that the enzymes responsible for protoporphyrin synthesis are present in constant concentrations in the cytoplasm the quantity of cytoplasm will determine the grain count per cell If the erythroblast (early pronormoblast) has a grain count of about 300 then the pronormoblast will have a grain count of 100 to 200 late polychromatic normoblasts will have a grain count of 50 to 60 and the reticulocyte will have 40 to 60 In the reticulocyte this is after the period of initial labeling There are only 7 to 10 grains per cell in those reticulocytes which are directly labeled

C P Leblond How soon after injection are these counts obtained (except for the reticulocytes)? When you give erythropoietin does the time for the change in production of red cells fit with its effecting stem cells?

E L Alpen The nucleated cells are all labeled within the first two hours If one gives erythropoietin and then measures the iron incorporation you can not see a really appreciable change until 24 to 36 hours after erythropoietin injections have started One can never see immediately the effect of a single erythropoeitin injection

F Stohlman Jr Some of the changes in red cell production following bleeding cannot be explained by increased input from the stem cell alone In the rat the peak iron incorporation is normally reached in 60 hours In the bled animal this time is shortened to 48 hours Moreover we see a change in the reticulocyte values earlier than can be explained by stem cell proliferation

I want to make it clear that I am not trying to explain the entire regulation of red cell production by any one mechanism It is my contention that there is ample evidence to suggest that all changes in cell production do not necessarily proceed from the stem cell e g, the change in emergence time which Dr Alpen has observed in dogs and we in rats the appearance of Fe^{59} in reticulum cells 12 to 24 hours after its administration which Dr Alpen has described and which appears compatible with a death process

V P Bond Dr Alpen what is the magnitude *and time of occurrence of the reticulocyte* response following bleeding in your animals? Are these compatible with an effect at the stem cell level and constant generation times?

E L Alpen I don t have the data at hand We have *never done reticulocyte* counts very early The reticulocyte response doesn t really start until about 24 to 36 hours after bleeding

In answer to Dr Stohlman the weight of my argument for the entry of stem cells does not rest on all of the ancillary data I presented It rests on only one thing the difference in the rate of change in the *percentage* of labeled erythroblasts in both bled and control animals while the ratio of change of grain counts per cell is unaffected There is only one way that the percentage of labeled cells in the bled animals can go down at such a dramatically rapid rate by the entry of an unlabeled cell population into the sample There is no other explanation

F Stohlman, Jr. I did not mean to imply that stem cells do not increase The one thing that has bothered me most at this conference is the compulsion to explain any and all changes in the rate of cell production on the basis of one mechanism May be there is more than one mechanism How long does it take to get from the stem cell to the circulating reticulocyte? I am certain in the dog it is over 24 to 36 hours

E L Alpen Yes it is longer than that In my paper, in which I was able to go into this in a little more detail, I have mentioned that I have not studied the first three days after bleeding very carefully A dog cannot be bled half of his blood volume in one day One gap that must be filled is what happens after one single severe bleeding I don t propose this as the only answer but that it is only one of the principal mechanisms I don t think one can account for all of the observed reticulocyte responses on the basis of our model There may be another direct action which causes the early release of reticulocytes

L Lamerton I think it is possible that if the number of early forms increases the reticulocytes will be pushed out quite quickly, just as when the population of early forms is depleted there appears to be a holdup in the release of the late forms Whether this is a mechanical holdup due to growth pressure I don t know If this were the case a normal population of early forms would have to be established before the rate of release was achieved

H Quastler Following irradiation you observed that the label came out faster Is this true when the blood count was about normal?

L Lamerton This has not been checked yet

H Quastler If you have a situation where you have had overproduction and stop it, as Dr Alpen proposed by shunting into the periphery a few erythroblasts which normally would divide a few more times it would mean that the iron which normally appears in the periphery only after several mitoses now emerges earlier

E L Alpen This is not the case In my model (*cf* figs 5 and 6 Alpen and Cranmore, page 297) the proportion of cells maturing from erythroblasts without division is always constant and unaffected by the proliferative state of the marrow The absolute number of cells which should come through without division depends on the marrow volume Thus, the proportion of iron going into the shunting' cells would be constant Assume that normally 5 per cent of the erythroblasts mature without dividing Following maximum marrow stimulation the blood volume returns to normal and erythropoietin production stops Then the rate of differentiation of stem cells would be reduced but the rate of shunting maturing erythroblasts is still 5 per cent Admittedly it is 5 per cent of an enlarged population but this does not change the rate of appearance of iron Five per cent of the total iron still emerges through this same process

H Quastler It would appear to me that erythroblasts would respond to some signal which says Don t divide, mature in which case the rate at which these cells emerge would be affected and hence the rate of appearance of iron

E L Alpen This bypassing is a normal process The fraction of cells affected is a constant no matter what the state of activity of erythropoiesis A return from a stimulated to a normal state occurs by virtue of a decrease in the number of stem cells differentiating into erythroblasts No signal is required to stimulate or depress this process

L G Lajtha I think the argument between Drs Stohlman and Alpen is a question of degree rather than kind I don t think that anybody would suggest that there is no cell death in the erythroid series I gather from Dr Stohlman s argument that he implies that this is a rather large factor which normally destroys a fair amount of the early erythroblasts to such an extent that after bleeding stoppage of cell death enables the duplication and quadruplication of the number of normoblasts This would imply that normally cell death is a very large factor—four times the actual number of normoblasts present otherwise you could not by stopping it increase the numbers four times I don t think there is any evidence for such a degree of cell death

F Stohlman Jr First we must remember that the bone marrow may be called on to replace less than one-half the blood volume, occasionally as little as 1, 2 or even 5 cc Is this accomplished by increasing stem cell differentiation? Is it not reasonable to suggest that there may be other mechanisms? Perhaps one for relatively minor blood loss and another for panic situations such as major blood loss an extreme of this being the loss of one half the blood volume? As I have said before there are probably multiple mechanisms

Now assume that normally 1 per cent of nucleated red cells die each day assume four divisions occur between erythroblast and reticulocyte so that erythroblasts make up only 3 per cent of nucleated red cells if all cell death occurred amongst erythroblasts then you could have a 50 per cent increase in output if this sort of death ceased I don't mean to imply however that this is the *only way* of increasing red cell output

G **BRECHER** If the proportion of all cell types is constant under all circumstances and the time they spend in transit through these compartments remains constant then the reticulocyte count in the peripheral blood should at all times give a perfect measure of red cell production If not either you don't have a steady state which I think is the best possibility because of the reticulocyte peaks or the relative proportions don't remain constant

E L **ALPEN** The relative proportions do stay constant except that stimulation initially increases the number of erythroblasts relative to the later forms We would predict this because in an expanding bone marrow the influx of stem cells would first increase the number of erythroblasts the erythroblasts would stay a little bit ahead until the steady state was reached The reticulocyte number would tell you something about production only in a steady state which we never achieve The response to bleeding is a rapid peaking of activity followed by a rapid fall off Hence we are on either the upslope or the downslope The relative number of reticulocytes in marrow and blood is another thing that must be taken into consideration We know that the proportion of reticulocytes in blood increases following bleeding The reticulocyte count therefore is only a measure of production in a steady state

F **STOHLMAN** JR. It seems to me in Dr Erslev's experiments the reticulocytes appeared in the periphery earlier than can be explained solely by stimulation of stem cells If one assumes that responses are due solely to changes originating in the stem cell then the administration of colchicine—which affects only mitosis and not release—would not result in a prompt fall in reticulocytes

A **ERSLEV** I don't think there is a basic discrepancy In the rabbit the time for maturation from the early stages until the release from the bone marrow is probably less than 48 hours The rate of Fe^{59} incorporation and reappearance in circulating red blood cells supports the thesis that nucleated red cells spend a very short time in the marrow Undoubtedly when you administer an anoxic stimulus there is an earlier release of reticulocytes from the bone marrow than normally In acute blood loss anemias for example unusually immature reticulocytes are released into the circulation possibly squeezed out by increased pressure in the bone marrow However the time spent from stem cell differentiation through three to four mitotic divisions until the cell reaches the reticulocyte stage is probably of the order of 24 to 48 hours

E L. **ALPEN** My iron studies show evidence of destruction of red cell precursors 12 to 24 hours after the administration of iron At this time one sees labeled reticulum cells in the bone marrow I think that these cells using Dr Bessis principle could account for the observed increase in iron incorporation that Dr Stohlman saw after the administration of altitude plasma

In conclusion I also should like to say that I don't feel that my model is the answer to the whole problem of red cell regulation although it answers the majority of problems I think most of the cell production in the severely stressed animal is by this mechanism but I also believe that the animal may have another regulatory mechanism which is responsible for handling a short term stress that will allow a more rapid proliferation under a very heavy stimulus

In this regard Dr Lajtha and I injected one rabbit with thymidine very early after bleeding and found an increased percentage of positive basophilic normoblasts which would indicate a shortened cell cycle (generation time *Ed*) This would seem to indicate that very early after bleeding some other mechanism is acting

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Discussion of Identity of Stem Cell and Site of Action of Erythropoietin

J M YOFFEY I was interested in Dr Alpen's conclusion that the increased erythropoietic activity involved the stem cells Our group has noted erythropoietic stimulation in response to anoxia The marrow erythroid population had to increase appreciably before new cells were discharged into the blood We then looked for stem cells Such a search can be approached in two ways. You can just talk about stem cells and never try to identify them If you do that you go in for some sort of hematologic mysticism Or else you can look at the marrow and try to find a stem cell I'm sorry to be boring about this I know a lot of you think 'This chap has got a bee in his bonnet. However the only other cell we found increased during this critical early period was our small lymphocyte

A second situation in which we looked for the appearance of stem cells was that following irradiation We gave an animal a sublethal dose of irradiation and then examined the marrow quantitatively at intervals We found that before there was significant red cell or granulocytic regeneration small lymphocytes in the marrow increased two and a half times In our normal guinea pig marrow we get something like 4×10^5 small lymphocytes per mm^3 Following irradiation the small lymphocytes increased to 1×10^6 That was the only stem cell that we could find If anyone can show me another stem cell I shall be very glad to see it

E. L. ALPEN I previously said that we propose that erythropoietin does not increase the number of divisions In fact it does nothing to the stem cell except direct the differentiation Now let us assign to the stem cell a pluripotential function Incidentally, the nature of the stem cell is not germane to the argument Presume for the moment that the reticulum cell or hemocytoblast is the stem cell for the erythroblast and that it is also the stem cell for epithelial cells lymphoblasts etc If we have a sudden need for production of erythrocytes the only thing that need be done is to salvage some stem cells that otherwise might degenerate into late reticular forms and direct the differentiation of these cells into erythroblasts The rate of division of stem cells need not be increased nor is an increased number of stem cells necessary The number of erythroblasts is not large and doubling or quadrupling their number does not require an increase in the number of stem cells which are already numerous enough

L O JACOBSON In transfusion induced polycythemic mice a reticulocyte response begins approximately two days after erythropoietin injection This agrees essentially with data that Dr Alpen and others presented on blast to reticulocyte time

I should also like to present an experiment we are currently doing When one transfuses a mouse with homologous red cells and elevates the hematocrit to about 70 per cent, red cell production goes to zero This polycythemic mouse is now irradiated with a lethal dose of x irradiation and given rat bone marrow The polycythemia is maintained by administering homologous mouse red cells as necessary to maintain elevated hematocrit Thus no erythropoiesis is initiated whereas the mouse makes rat leukocytes and platelets After about 25 days the red cell mass is brought down and the mouse immediately begins to make *rat red cells* We may ask ourselves the question From which precursor or cell type did the rat red cells arise? Either multipotential cells began production of rat red cells when the stimulus for red cell production was reintroduced or differentiated rat red cell precursors lay dormant in the marrow during this three week period

E L Alpen This is a very beautiful observation It might provide a way to get at the stem cell question The hypertransfusion aplasia of bone marrow does tie in with this The fall off of production of bone marrow following hypertransfusion does check in time with our other observations the bone marrow activity reaches its low point in two and a half days

J M Yoffey We do not find a great reticulocyte store in the marrow of the guinea pig We find in the blood about $7\ 5 \times 10^{4}$ reticulocytes per mm^{3} and an absolute marrow count of $2\ 5 \times 10^{5}$ reticulocytes There is not a big reserve of marrow reticulocytes

E L Alpen In man and dog the number of reticulocytes within the bone marrow is equal to the number of nucleated cells

J M Yoffey This is very pertinent Our nucleated red cells in the guinea pig marrow are 4×10^{5} reticulocytes $2\ 46 \times 10^{5}$ If you discharge all those reticulocytes we still wouldn t have a large number for rapid addition to the blood our marrow volume is 6 2 cm^{3} and our blood volume is 28 cm^{3}

E E Osgood Dr Alpen what proof is there that the cell of origin is a multipotential cell? Couldn t it be simply a blast cell that is already definitely going to produce cells of the erythrocytic series but has not yet developed the capacity to incorporate iron? We have cultures containing erythrocytic cells which we could get to grow only when there were definitely nucleated erythrocytes present in the blood from which the culture was derived Perhaps it is a question of semantics but I can not see why an alpha 2-alpha division in a rubriblast or prorubricyte that was definitely of the erythrocytic series but not yet incorporating iron wouldn t explain these results equally well

E L Alpen I have avoided completely the question of what is the stem cell I just wrote stem cell on the slide and don t propose to get into any arguments about what it is at this point I don t consider the point productive But if it was already a differentiated erythroid cell we would see in the control dog a dilution of the labeled erythroblasts by these unlabeled cells We don t see this Perhaps this is because it is a very small number in the control but much greater in the bled animal We do know that in the bled dog there is something entering the erythroblast pool It may be a lymphocyte as Dr Yoffey proposes a primitive progenitor cell as Dr Cronkite calls it a stem cell as I call it or whatever you may happen to call it

L G Lajtha In reference to Dr Gordon s paper Dr Alpen mentioned that we have tested Dr Van Dyke s preparation of erythropoietin in human marrows in vitro measuring several parameters—DNA synthesis rate of cell proliferation iron uptake—and found no effect This on the face of it fits nicely with the concept of the regulation of erythropoiesis which Dr Alpen proposed I do not see any principal contradiction between these findings and those of Dr Gordon First of all it is possible that this substance in the urine is in a conjugated form of some sort and has to be deconjugated which happens in vivo But when we look at Dr Gordon s figures one wonders whether this is a direct effect on the proliferation of cells already differentiated into erythroid forms or an indirect action on a stem cell Whichever way I look at it no explanation seems possible If it is through a stem cell the implication would be that within four hours a large number of stem cells would have to rush through to polychromatic normoblasts because for the most part these are the cells which Dr Gordon counts The time is far too short for such developments If the material is acting on mitosis then it would imply that a whole population of relatively late normoblasts would have to double We know the normal mitotic index rate of DNA synthesis per cell and proportion of cells in DNA synthesis for the late normoblast is extremely low There would have to be a complete cell cycle for every one of them within four hours It doesn t make sense Nevertheless the facts are there and I suggest that these facts not only raise the question of the mechanism of erythropoiesis but also raise very basic questions in general cell physiology It is extremely important to study the mechanism of processes occurring in Dr Gordon s preparation

A S Gordon We are also concerned

about the rapidity with which the nucleated RBC increases occur in the isolated hind leg preparation perfused with material containing erythropoietin The factor may act, in the isolated perfused preparation, by provoking mitotic division, particularly at the level of the polychromatic normoblast In the perfusion studies, we have accumulated some evidence for the conversion, under conditions of erythropoietic stimulation, of lymphocytes ("small round cells") into members of the nucleated RBC series, as proposed by Dr Yoffey But such transformation would have to proceed in a greatly speeded fashion to account for the increased numbers of hemoglobin containing RBC within the marrows of hind legs perfused with urinary erythropoietin for *the short time of a four hour perfusion* Another possibility is that the factor operates by decreasing the rate of cell death in the marrow We propose to test these possibilities with radioisotopic methods It should be remembered that we are dealing here with a limb and its contained marrow separated from the rest of the body It is conceivable that under these conditions, divorced from any possible restraining influences which might be exerted by other organs, hemopoietic processes may proceed *in a manner quite different from* what is seen in the intact *organism* Whatever the explanation may be, the bald fact remains that marked increases in the absolute numbers of hemoglobin bearing nucleated RBC occur in the marrows of limbs perfused for four hours with blood to which urinary erythropoietin has been added

V R Potter Dr Gordon, is it possible to detect a decrease in any other class of cells? Is the total number of cells so great and this particular fraction so small that one is not in a position to find out where they are coming from?

A S Gordon The counts we make are expressed in absolute numbers, not in percentages The nucleated RBC of rat marrow comprise about 25 per cent and the granulocytes about 50 per cent of the total nucleated cells There is generally little change in the total nucleated cellularity of the femoral marrow after four hours of perfusion The increases in the numbers of nucleated RBC evoked by erythropoietin are usually associated with decreases in the numbers of granulocytic and occasionally lymphocytic elements It would almost seem that stimulation of the production of one cell type in the perfused leg demands that production of other cell types be simultaneously depressed

V R Potter I think there is reason to describe differentiation in general as induction of a specific enzyme in a cell *which has the potentiality for it but lacks* actual amounts of enzyme, yet which must have had the enzyme-forming system There is a very useful technic which has been used *not only in bacteria*, but in mammalian tissues, namely, the prevention of enzyme induction by the administration of ethionine In a situation such as this one could administer ethionine and perhaps block the conversion of this stem cell into the cell which possesses the necessary enzyme and is therefore by definition now a *differentiated* cell Perhaps one could use colchicine and ethionine as two complementary tools, one of which would block cell division and the *other differentiation* (differentiation being defined in terms of the production of the specific enzyme) Ethionine has been used in intact animals, and therefore I think it ought to be possible in a perfused limb If there is an effect, then you could administer methionine in the presence *of ethionine* and it should restore the ability to differentiate I liked Dr Alpen's general suggestion very much and I think it makes a great deal of sense that is, *conversion* of a potential cell into another without the occurrence of cell division at this point

Properties of the Urinary Erythropoietic-Stimulating Factor (ESF)

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THE erythropoietic stimulating factor (ESF), also termed hemopoietin(e) or erythropoietin(e), has been demonstrated unequivocally only in blood and urine In this regard, blood has been extensively investigated, and the more constant sources thus far uncovered have been plasma or serum from animals subjected to anemic or hypoxic anoxia and from anemic humans [2,3,7,16]

It is only recently that attention has been given to the urinary excretion of this factor Hodgson and Toha[13] observed that the urine of repeatedly bled rabbits sped regeneration of hemoglobin in rabbits subjected to a standard hemorrhage The first indication of erythropoietic stimulating activity in human urine was noted in a case of Cooley s anemia [19] Following this observation several reports appeared demonstrating the existence of the ESF in the urine of patients with Cooley s anemia,[10,27,28] hypoplastic anemia,[10,11,25,27] leukemia associated with anemia,[10,18,23] and in the urine of rabbits rendered severely anemic with phenylhydrazine [12] Urine from normal animals or human subjects possessed no erythropoietic stimulating activity [10,12,13,27]

The present paper describes the incidence of occurrence of the urinary ESF in patients with Cooley s anemia† and delineates some of its biologic and chemical properties

RELATION BETWEEN THE DEGREE OF ANEMIA IN COOLEY S ANEMIA SUBJECTS AND THE PRESENCE OF THE ESF IN THEIR URINE

Previous studies[10,27] indicated that urine from children with Cooley's anemia was a less constant source of the ESF than plasma from the same patients At that time, no relation could be drawn between the presence of the ESF in urine and the intensity of the anemia or the degree of concentration (specific gravity) of the urine More recently, we have extended our studies of the urinary ESF in Cooley's anemia to include 13 patients who contributed the 30 urine samples tested All urines were collected over a two- to three-day period prior to the day of transfusion The specimens were kept under refrigeration during the time of collection and for the experimental period thereafter The method employed for screening the urine samples for erythropoietic stimulating activity consists in

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TABLE 1 —*Relation of Intensity of Anemia in Subjects With Cooley s Anemia to the Incidence of ESF Activity in Their Urine*

No samples	Donor Hb range (Gm. %)	% positive responses in recipient rats
10	3 5—4 8	90
10	4 8—5 9	80
10	6 0—12 0	20

injecting subcutaneously 3 ml of the filtered unconcentrated urine daily for five consecutive days into a minimum of three adult female rats of a modified Long Evans strain * Peripheral blood hematocrit and reticulocyte determinations are made prior to the period of treatment and on the day following the fifth injection An increase in reticulocyte numbers accompanied by no decrease or preferably an increase, in hematocrit values constitutes a positive response Reticulocyte increases associated with a fall in hematocrit are interpreted as due to lytic effects and the response is not deemed positive

It will be noted from table 1 that 9 of the 10 samples obtained from patients with hemoglobin levels ranging from 3 5 to 4 8 Gm per cent elicited positive erythropoietic stimulating effects in the recipient rats Samples from patients with hemoglobin values ranging from 4 8 to 5 9 Gm per cent were 80 per cent effective, whereas a low incidence of activity was observed in the urines from patients with hemoglobins above the 6 0 Gm per cent level It would thus appear that a positive correlation does exist between the intensity of the anemia in the donor subject and the presence of the ESF in urine Our inability to demonstrate this relation previously was probably the result of not having sufficient numbers of samples from intensely anemic patients

EFFECTS OF A HIGHLY ACTIVE URINE ON THE TOTAL NUMBERS OF RBC

Table 2 indicates the marked effects of a highly active urine sample (TR 1) obtained from an 8 year old boy with Cooley s anemia (hemoglobin—3 8 Gm per cent) on the hematocrit and reticulocyte values of rats Attempts were made to determine the minimal effective dose of this urine with our standard assay procedure Four groups of three rats each were injected daily with quantities of the urine ranging from 0 5 to 3 0 ml per day for five days Figure 1 indicates that proportionately greater rises in reticulocyte and hematocrit values occurred with increasing amounts of urine up to 2 0 ml per day On this particular occasion, the 3 ml daily dose was less effective in augmenting these parameters than the 2 0 ml quantities probably because of some superimposed toxicity manifested by nonerythropoietic components of the urine at the higher dose level

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* In almost all cases the human urine was well tolerated by rats of our colony No untoward symptoms developed after administration of as many as 20 daily injections of active urine Moreover, the urine treated rats displayed body weight gains comparable to those experienced by untreated controls The toxic effects observed only occasionally may possibly be the result of larger amounts of nonerythropoietic lytic components of urine or to the presence of antigenic material in these particular urine specimens

TABLE 2—*Effects of 5 Daily 3 ml Injections of Urine From a Child with Cooley's Anemia (TR 1) on Reticulocyte and Hematocrit Values in Rats*

Rat no	Ret cs %		Hematocrit %	
	B	A	B	A
1	2.3	12.0	49.5	50.6
2	3.9	12.4	45.2	53.9
3	3.1	14.2	43.9	52.5
Mean	3.1	12.9	46.2	52.3
S E $_m$	.46	.68	1.7	.96
P	<0.001		<0.01	

B—value before treatment
A—value day after last injection

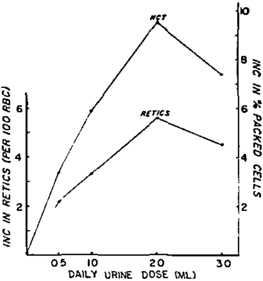

FIG 1.—Erythropoietic response to four dose levels of active untreated urine from a child with Cooley's anemia.

At other times when this urine sample was assayed, the 3 ml dose did not reveal this diminished effectiveness.

It seemed important to determine whether administration of this urine would produce true polycythemia in recipient rats. To this end, the labeled RBC dilution method of Berlin et al.[1] for estimating total circulating RBC volume was modified for determination of the total RBC numbers. Donor rats received 20 μc Fe^{59}Cl$_3$ intraperitoneally. A week later, the donor RBC were washed to constant activity with plasma and a sample administered intravenously into five adult female rats which previously had been injected daily for 11 days with 3 ml of urine specimen TR 1. The total activity (A_1) and the total number of RBC injected (R_1, determined with a Coulter electronic counter) were ascertained. Similarly, the activity per RBC(a/r) was estimated for the blood of the recipient

TABLE 3.—*Effects of Untreated Urine From a Child With Cooley's Anemia (TR 1) on the Absolute Numbers of Circulating RBC in Rats*

	Billions RBC per 100 Gm. Body Weight	
	Controls	Urine treated
	52.0	61.0
	51.5	62.2
	46.0	56.8
	54.4	64.6
	53.1	69.6
Mean	51.4	62.8
S.E.$_m$	1.3	2.1
P	<0.01	

rats after random distribution of the cells in the circulation. The total number of circulating RBC(R) was calculated from

$$R = \frac{I_t}{a/r} - R_t$$

The values for the five rats treated with active urine TR 1 and those for five untreated rats are given in table 3. It is apparent that the urine produced a highly significant augmentation in the absolute numbers of RBC, in this case amounting to 11.4 billion cells per 100 Gm. body weight, or approximately a 20 per cent increase. Hematocrit values were augmented in the urine treated rats from a mean of 47.5 to 57.8 per cent, also an increase of approximately 20 per cent. This would tend to indicate that the erythropoietic stimulation caused by the active urine is associated with the production of normal sized RBC. Unlike dye-dilution procedures for blood volume determinations, total RBC values determined with this technic do not vary within one to five hours after injection of the labeled cells. The method is independent of the state of hydration of the animal and local variations in tissue RBC concentration, and depends solely on random mixing and counting statistics.

NATURE OF THE RETICULOCYTE RESPONSE

Additional evidence for enhanced RBC production in rats treated with active urine is seen from the intensity of the reticulocyte response, characterized by increased numbers of immature forms. Thus, as seen in table 4, daily subcutaneous

TABLE 4.—*Heilmeyer Classification of Reticulocytes in Blood of 3 Untreated Rats and 3 Rats Injected Daily for 5 Days With 3 ml. of Urine From a Child With Cooley's Anemia (TR 1). Mean % values (±S.E.) for each category are given*

	Class			
	0 + 1	2	3	4
Controls	5 ± 0.22	23 ± 4.3	26 ± 2.7	46 ± 1.9
Urine treated	21 ± 1.5	36 ± 2.9	19 ± 3.9	24 ± 0.59
P	<0.01	<0.1 >0.05	0.2	<0.01

injections of 3 ml of sample TR 1 for 5 days resulted in a pronounced shift to the left in the Heilmeyer Trachtenberg formula[22] It will be noted that whereas only 28 per cent of the reticulocytes in the blood of control rats were in Classes 0, 1, and 2 (most immature types), there were 57 per cent of this variety in the urine treated animals These observations further strengthen the view that such urine possesses marked erythropoietic stimulating qualities

PROPERTIES OF THE RBC *PRODUCED UNDER THE STIMULUS OF THE* URINARY ESF

Linman and Bethell[15, 16] have repeatedly shown that although their boiled filtrates of plasma from anemic rabbits and polycythemic patients induce marked elevations in the numbers of RBC accompanied by intense reticulocytosis and myeloid erythroid hyperplasia in rats, the erythrocytosis is apparently the result of increased production of short lived microcytes and is not associated with increases in hemoglobin values It was deemed important to determine whether abnormalities also existed in the RBC produced in response to urinary ESF

(a) *Hemoglobin studies* It was possible that administration of the urine had resulted in the formation of a defective hemoglobin and that the ensuing erythrocytosis was a response engendered to compensate for this functional abnormality The methods of alkali denaturation and electrophoretic mobility delineated by Wintrobe,[29] were employed to test this possibility For alkali denaturation blood was obtained from three rats injected daily for 17 days with 3 ml of urine TR 1 No significant differences were encountered in the percentages of hemoglobin F in this blood, as compared to samples obtained from three untreated control rats

In the electrophoretic mobility analysis, hemoglobin was obtained by hemolyzing the cells with distilled water and toluene[29] 6 μl of approximately an 8 per cent solution of the hemolysate was applied to paper strips in a Spinco Model R paper electrophoresis cell Spinco B 1 buffer was used and four hours were allowed for migration under the influence of a constant current of 11 ma per cell The positions, shapes, and sizes of the spots for the hemoglobins of three rats treated for two weeks with active urine were no different from those of three controls No differences in mobilities were apparent even after an additional eight hours were allowed for migration

(b) *RBC resistance* Osmotic fragility determinations were conducted on blood from untreated controls and from rats treated daily for 17 days with 3 ml of urine TR 1 Todd and Sanford s procedure[24] was followed Figure 2 indicates that no significant differences exist between the lysis curves for the bloods of two urine treated rats and a representative control

(c) *Functional behavior of the RBC* Additional evidence for the competency of the RBC in rats receiving urine from Cooley's anemia patients is revealed in the changes of the reticulocyte levels following two successive bouts of treatment To the left of figure 3 are the alterations in reticulocyte numbers after the first series of 12 daily 3 ml injections of an erythropoietically active urine (PA 3) into three intact rats The rise in reticulocyte numbers during treatment is plain Of special significance is the descent in the reticulocytes to levels below normal a few days after cessation of treatment This ability of the RBC, formed in excess numbers in response to the urinary ESF, to curtail RBC production, speaks for their normal oxygen carrying capacity. Depicted to the right of figure 3 is the reticulocyte response in the same three animals to a second series of 12 injections of the urine. It will be noted that the magnitude of the initial phase of the response is similar to that seen in the first series of injections, but that the level tends to fall off after a peak reaction attained at six days.

Thus, from the hemoglobin, osmotic fragility, and reticulocyte behavior stud-

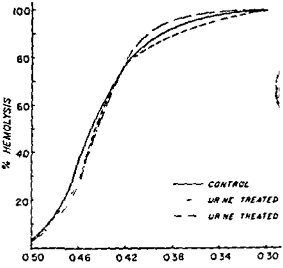

FIG 2.—Osmotic fragility curves for blood from a representative untreated rat and from two rats receiving erythropoietically active urine from a child with Cooley's anemia.

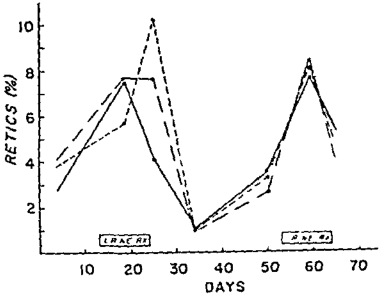

FIG 3.—Reticulocyte behavior during and in between two bouts of treatment with urine from a child with Cooley's anemia.

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ies, it would seem that the RBC evoked by the urinary ESF constitute a functionally competent population of cells. This is unlike the situation described by Linman and Bethell in their rats receiving boiled filtrates of plasma from anemic animals or polycythemic patients.

INFLUENCE OF THE URINARY ESF AND THE HYPOPHYSEAL GROWTH HORMONE ON ERYTHROPOIESIS IN PERFUSED ISOLATED HIND LIMBS OF RATS

We have previously described a technic for perfusion of isolated hind limbs that permits the determination of hemopoietic activity within the contained bone marrow.[8, 10, 14] Perfusion of normal rat limbs with blood from normal rats did not alter significantly the absolute numbers of marrow nucleated RBC, when compared to those in the marrows of the contralateral nonperfused limbs. However, increases in the absolute numbers of nucleated RBC occurred within the limbs perfused with blood from low pressure–exposed, bled, or phenylhydrazine-treated rats. Significantly increased numbers of mitotic figures were evident among the members of the erythroblast series, especially in the marrows of limbs perfused with blood from repeatedly bled or phenylhydrazinized rats. It was concluded from these experiments that the ESF present in the blood of animals subjected to various forms of hypoxia exerts its stimulatory effects directly upon the blood forming organs.

It was of interest to determine whether the urinary ESF was also capable of stimulating erythropoiesis in the isolated limb preparation. In the first series of experiments, hind limbs of normal adult rats were perfused with 50 ml. of normal rat blood to which was added 1 ml. of active unconcentrated urine (TR 1) over the four hour period of perfusion (0.25 ml. per hour). The numbers of benzidine-positive nucleated RBC per mg. of marrow[5, 10] were estimated in the femoral marrows within the control contralateral limbs at the beginning of the experiment, and then in the limbs perfused for the standard four hour period. Table 5 shows that 1 ml. quantities of urine exerted no marked influence on the numbers of nucleated RBC in the marrows of perfused limbs. When the amount of urine perfused was raised to 4 ml. (1 ml. per hour), however, a mean increase of 84 per cent in nucleated RBC numbers over that of the nonperfused limbs was evident.

In view of the greater sensitivity of the erythrogenic marrow in hypophysectomized animals to hormonal stimulation,[4] a second series of experiments was conducted in which hind limbs from animals hypophysectomized two weeks previously were perfused for four hours with approximately 50 ml. of hypophysectomized rat blood, with and without the urinary ESF. The two week posthypophysectomy period was selected because, at this time, the bone marrow exhibits a maximal depression of erythropoiesis. Table 5 indicates that perfusion of hypophysectomized rat limbs with blood from hypophysectomized rats containing no added urine, resulted in no significant change. On the other hand, a mean increase of approximately 120 per cent in the numbers of nucleated RBC occurred in the marrows of hypophysectomized animal limbs perfused with blood from hypophysectomized donors to which 1 ml. of active urine had been added (0.25 ml. per hour). Addition of 2.0 ml. of urine over the four hour period (0.5 ml. per hour) led to no discernible change in nucleated RBC numbers, probably because of toxic

TABLE 5—*Effects of Urine From a Child With Cooley's Anemia (TR 1) and Hypophyseal Growth Hormone (GH) on Erythropoiesis in Perfused Isolated Rat Limbs Figures Represent Thousands of Nucleated RCB per mg of Femoral Marrow*

Animal No	Substance Added	Limbs from intact rats perf with normal rat blood	
		*Control**	*Perfused†*
B46	none	731.4	654.7
B48	none	788.6	836.0
C10	none	400.5	452.0
C13	none	492.1	537.3
C19	1 ml TR 1	629.8	655.4
C24	1 ml TR 1	489.9	556.2
C20	4 ml TR 1	336.8	630.3
C25	4 ml TR 1	364.5	665.2
		Limbs from hypx. rats perf with hypx rat blood	
		Control*	Perfused
B74	none	122.5	103.7
C7	none	55.1	51.4
C15	none	71.3	90.6
C18	1 ml TR 1	33.9	165.7
C22	1 ml TR 1	64.1	264.0
C23	1 ml TR 1	104.0	175.0
C21	2 ml TR 1	75.0	73.4
B75	2 mg GH	104.9	137.2
C8	2 mg GH	55.4	122.1
C16	2 mg GH	110.2	156.6
C17	4 mg GH	90.4	81.6

* Control—Contralateral nonperfused limb

† Perfused—Perfused limb

pecially to those of adenohypophyseal thyroidal or adrenal cortical origin to which the erythropoietic process in hypophysectomized rats is particularly responsive Some of these may be acting in synergistic combinations Since the growth hormone (GH) has been demonstrated to be a potent stimulator of marrow erythropoiesis in hypophysectomized rats [9] and since it does not appear to require an intermediate endocrine organ for some of its actions we have initiated experiments dealing with its effects on erythropoiesis in isolated limb preparations As in the above experiments hind limbs of rats hypophysectomized two weeks previously were perfused with approximately 50 ml of blood from hypophysectomized donors Addition of 2 mg GH* at the beginning of the experiment resulted in a mean increase of 64 per cent in the numbers of nucleated RBC in the marrows of three hypophysectomized rat limbs studied after four hours of perfusion This effect which was not as marked as the response elicited with 1 ml of active urine was nevertheless consistently present (table 5) Addition of 4 mg GH to the perfusate led to no change in the numbers of nucleated RBC in the perfused limb In fact this quantity of GH was decidedly toxic to the marrow with unmistakable signs of destruction of many of the marrow cellular elements obvious at the termination of the perfusion period

These experiments indicate that the erythropoietic stimulating activity of GH noted in the hypophysectomized rat is also evident in the perfused hind limb preparation and this strengthens the view that the action of GH on erythropoiesis is a direct one At the same time the results suggest that caution be taken in interpreting erythropoietic stimulation in hypophysectomized rats as due solely to the ESF content of the material being tested For this reason despite its relative insensitivity, the intact animal marrow appears in the final analysis more desirable for assay purposes because of its greater degree of specificity to the ESF

Chemical Properties of the Urinary ESF

At present opinion is varied concerning the chemical nature of ESF Evidence has been adduced that the ESF appearing in the plasma of rabbits made intensely anemic with phenylhydrazine is a mucoprotein or an active grouping associated with it [6, 10, 12, 17, 20] Some regard it as most likely a polypeptide [23] while others[16] hold that two factors exist one thermostable and lipid in nature which stimulates marrow erythroid hyperplasia and the second thermolabile presumably a protein that augments hemoglobin synthesis

We have previously demonstrated[10, 23] that the ESF in urine from children with Cooley s anemia can be adsorbed on kaolin in the acid range A pH of 4 8 gives somewhat better results than the other pH s tested Serial elution carried out in the alkaline range with ammonium acetate buffers of increasing pH and with 1M NH_4OH revealed the greatest activity to be in the pH 8 and 1M NH_4OH eluates Calculations indicated that a 39 per cent yield with a 230-fold purification of the urinary factor was obtained in the 1M NH_4OH eluate The purified preparation produced metachromasia with toluidine blue had an absorption peak at 280 mμ, migrated very slowly in the paper electrophoretic system and showed little displacement of the boundary at 145,000 × g for 2 hours Injections of 300 μg a day for 5 days of the 1M NH_4OH eluate significantly stimulated erythropoiesis in intact rats With a more sensitive technic, such as Fe^{59} incorporation into the circulating RBC of starved rats, approximately 60 to 75 μg per day would be required to elicit a positive response

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* Dr S L. Steelman of the Armour Laboratories supplied the beef growth hormone (Armour No M 108) This preparation when administered in daily 1 mg quantities to hypophysectomized rats averaging 180 Gm. in body weight resulted in body weight gains of 30 ± 4 Gm over a 7-day period

Our analysis of the 1M NH_4OH eluate of urine from Cooley's subjects indicate a carbohydrate content of 23 per cent (anthrone reaction) Van Dyke et al[26] have stated that residues of ultrafiltered urine, preparations they have employed, contain approximately 50 per cent carbohydrate It is possible that the alkali used for elution in our procedure[10, 23] has resulted in separation of a considerable amount of carbohydrate from the moiety In this connection the electrophoretic mobility of our urinary material is lower than that of active plasma preparations which have been reported to migrate as alpha globulins More recently, Rambach et al[21] noted a decrease in paper anodic mobility of the plasma ESF with the cleavage of carbohydrate from the material Our purified urinary preparation appears to be material from which considerable electro negative carbohydrate has been separated The possibility therefore exists that the alkali in our kaolin procedure serves as an aid in the reduction of the moiety to its smallest possible effective size At the same time we have noted that a turbidity eventually develops after eluting the factor with 1M NH_4OH from its adsorbed state on kaolin This probably represents denaturation and is associated in our experience with variable degrees of loss of activity It seems likely that differences in the amount and type of carrier, characteristics that probably vary from preparation to preparation, may account for differences in the extent of the denaturation process and the reduction in activity noted with some urine samples subjected to the kaolin adsorption alkali elution procedure

Although our experiments show that not all the carbohydrate present in the urine specimens is essential for activity, they do not completely preclude the possibility that some minimal amount of carbohydrate in the structure is required Further experiments, preferably with the neuraminidase of influenza virus origin, would be needed to determine the essentiality of a carbohydrate component for activity. On the other hand, the importance of polypeptide groupings is supported by our findings[10] that erythropoietic stimulating activity of urinary ESF is destroyed after incubation with trypsin or chymotrypsin Van Dyke et al[26] who have used the residue of ultrafiltered urine from a patient with aplastic anemia, observed only partial inactivation of the urinary ESF with trypsin However, their enzyme substrate ratio was lower than that employed by us Indeed, our enzyme data are in agreement with those reported by others for the plasma ESF[17, 21]

Summary

1 A positive correlation exists between the intensity of anemia in Cooley's anemia subjects and the presence of ESF in their urine

2 Injections of erythropoietically active samples of untreated urine from children with Cooley's anemia induce marked reticulocytosis and hematocrit increases in normal rats A true polycythemia results, as evidenced from measurements of the absolute numbers of RBC within the recipients

3 The RBC of rats receiving urinary ESF show no increase in the amounts of abnormal hemoglobin as demonstrated by alkali denaturation and electrophoretic procedures Osmotic fragility and functional activity of the RBC also appear normal

4 Erythropoiesis is stimulated in the marrows of isolated hind limbs of rats to which the urinary ESF has been added Hypophysectomized rat limbs respond more sensitively to the urinary ESF than do limbs from intact animals Hypophyseal growth hormone also increases the numbers of nucleated RBC in perfused hypophysectomized rat limb preparations

5 Recent evidence points to urinary ESF as a mucoprotein or an active grouping associated with it The question that remains to be answered concerns the essentiality of the carbohydrate moiety in urinary ESF The importance of polypeptide groupings is indicated from the loss of activity following incubation with proteolytic enzymes

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Control of Red Cell Formation

By Leon O Jacobson, E Goldwasser and C W Gurney

INVESTIGATIONS in many laboratories have established the validity of Carnot and Deflandre's[3] original proposal that erythropoiesis is controlled by a humoral mechanism It is the purpose of this communication to give the results of some of our investigations and those of others that were used to develop a working concept of the control of erythropoiesis The development of such a concept at this time involves considerable speculation because there are obvious gaps in our knowledge of the basic physiology of red blood cell formation

Concept of Erythropoietin Production

Our concept of the dynamic equilibrium of the erythron may be stated in simple terms The norm for each organism in terms of its general level of metabolism is probably determined genetically Thus, as development continues under normal conditions, a pattern of metabolic activities is established This is governed chiefly by the hypophysis, but other glands, such as the thyroid, adrenals, and gonads, also take part The pattern determines the O_2 requirement of the body, which in turn determines the size of the red cell mass that is needed to satisfy the O_2 requirement as growth proceeds The red cell mass of the adult is increased or reduced by fluctuations in the production of erythropoietin which occur in response to a sensitive regulator that monitors the delicate overall balance between tissue O_2 demand and supply

To state that under normal conditions the production of erythropoietin and erythropoiesis are regulated by this relationship is simply another way of saying that erythropoietin production is regulated by the response of a site in the body that is sensitive to changes in pO_2 This site may or may not be the tissue or organ of production The stimulus for the production of erythropoietin may not directly involve O_2 It may be that some metabolic product, which reflects the O_2 demand supply relationship for the body as a whole, excites production of the hormone

Bomford[1] has suggested that the anemia of hypothyroidism may be a reflection of a reduction in the demand for oxygen Reissmann[33] reported that the pO_2 might be of more importance than the arterial O_2 saturation in the regulation of erythropoiesis Our hypothesis is based on a number of studies, some of which were reinvestigations of known physiologic phenomena[4, 22, 40, 44] and some of which were new approaches to the problem [9, 10, 19, 25]

In table 1 are listed some conditions and a summary of some observations that led us to consider the ideas described above These conditions are but a few of many that alter the rate of production of erythropoietin

Since the mechanism of production of erythropoietin appears to be related to tissue pO_2, one would expect that stress situations, which the body regularly encounters and which would alter pO_2, would alter erythropoietin formation and

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TABLE 1—*Relationship of Oxygen Suppy and Demand to Erythropoiesis*

Condition	O_2 Supply	O_2 Demand	Rate of Erythropoiesis	Sensitivity to Erythropoietin
Hypophysectomy	Normal	Decreased	Reduced	Increased
Hyperoxia	Increased	Normal	Reduced	Increased
Starvation	Normal	Decreased	Reduced	Increased
Polycythemia*	Increased	Normal	Reduced	Increased
Anemia due to				
Phlebotomy	Reduced	Normal	Increased	Decreased
Phenylhydrazine	Reduced	Normal	Increased	Decreased
Hypoxic Hypoxia	Reduced	Normal	Increased	—
Dinitrophenol	Normal	Increased	Increased	Decreased
Triiodothyronine	Normal	Increased	Increased	Decreased

* Induced by red cell injection

thus erythropoiesis. However, the reflexes that set in action increased respiratory rate, increased heart rate, etc., as a result of these stimuli might be expected to partially or largely compensate for the changing pO_2. Failure to compensate would result in increased production of erythropoietin, which would be sustained under certain conditions, e.g., in ascent to higher altitude, or might cause a temporary spurt of production in an organism in which the compensatory mechanisms were only temporarily inadequate.

One can imagine many spurts in production in a single day, but over a 24 hour period or several days, these temporary or "false" starts would not materially affect the steady "need" aspect of production. The "need" production refers to that production that is required to keep the red cell mass from decreasing owing to the loss of approximately 1 per cent per day, by the death of red cells.

If methods of measuring the plasma erythropoietin titer were sufficiently sensitive, one would expect a sharp rise in the plasma titer of this hormone as the result of short spurts caused by intense physical activity. One would also expect the production of the hormone to be higher during the day, when activity is sustained, rather than at night, when basal conditions prevail. This suggestion is strengthened by the observations of others,[19, 20, 45] who found that the plasma iron was higher at night than during the day. As shown in table 2, plasma iron levels are reduced within four hours in the normal rat by acute hemorrhage or the administration of cobalt ion. These data suggest that an increased hormone pro-

TABLE 2—*Fall in Serum Iron Associated With Sudden Stimulation of Erythropoiesis. Value in Gamma Per Cent Is Average of 5 Rats (300 to 340 Gm female) Per Group*

Stimulus	Time		
	0 hr	4 hr	8 hr
Control (Saline)	253	264	290
Phlebotomy (3% of body weight)		131	64
Cobaltous ion (30 μM/100 Gm.)		222	162

duction occurs and that its action can be ascertained even before assayable plasma levels of the hormone are reached

We are currently investigating several aspects of this problem the extent to which dilution of the plasma by extracellular fluid of low iron content contributes to the acute lowering of the plasma iron after phlebotomy, the changes in iron binding capacity accompanying changes in plasma iron, the effect of erythropoietin on plasma iron, and the effect of abrupt intense physical activity on this phenomenon One might anticipate that the plasma iron level would fall if the rate of erythropoiesis increased It is quite possible that with a slight increase in the production of erythropoietin, increased erythropoiesis would be evidenced by a decrease in plasma iron much before a change in the number of reticulocytes would be recognized In fact, one might anticipate that the amount of erythropoietin produced upon stress might be so small that in vivo or in vitro utilization of iron would increase temporarily without significant production of new red cells Even with sensitive methods of assay, no measurable increase in the plasma level of erythropoietin might be observed, because of immediate and essentially complete utilization in this early phase of the erythropoietin response to stimuli If the rapid change we have observed in the plasma iron, following introduction of intense stimuli to erythropoiesis, is a manifestation of a rapid increase in utilization of iron by the marrow, then it must follow that the removal of iron from the plasma must exceed, at least for a time, the rate at which the iron stores supply iron to the plasma

Ascent in altitude can be simulated in a low pressure chamber In an individual subjected to this treatment, the compensatory mechanisms normally operating are unable to compensate sufficiently, and as a consequence, the level of erythropoietin rises and erythropoiesis increases The red cell mass is increased slowly to a value that supplies oxygen to the tissues at a rate similar to that at the original altitude On rapid descent to sea level, a sharp decrease in the plasma erythropoietin titer is observed, and erythropoiesis drops essentially to zero The red cell mass attained at high altitude provides a tissue oxygen supply that is in excess of the need for oxygen by the tissues at sea level As the red cell mass falls with the natural daily red cell loss, equilibrium with the new environment is again attained, and erythropoietin production and erythropoiesis are resumed to maintain a red cell mass consistent with the new steady state The condition that obtains in rapid descent from altitude is like the one that occurs in animals subjected to hypophysectomy,[9] hypertransfusion,[2a] starvation,[10] or admission to a high oxygen atmosphere[10] (table 1) The etiology producing these various conditions is obviously different, but as illustrated in table 1, the ultimate mechanism responsible for reducing erythropoiesis in each instance appears to depend on the relationship of tissue oxygen demand to supply

A discussion of the metabolism of erythropoietin involves several important considerations namely, what cell or cells constitute the site of action of the hormone, what action does the hormone have on these cells, is the hormone utilized completely in the normal process of stimulating erythropoiesis, or does degradation in the marrow or elsewhere occur in addition to utilization, and, finally, what role does urinary excretion play in the economy of this hormone? At the present time, we can present only a relatively few facts in attempting to answer these questions

The first of these considerations concerning the site and mode of action of the hormone raises the question of whether or not more than one erythropoietin exists, and if so, do these have different actions, as suggested by the data of Linman and Bethell[23, 24] We are inclined, for the present, to disregard these data for the sake of simplicity and assume that only one erythropoietin exists in the normal state There are in point of fact, data to support this latter concept, since, in our own experience and in that of others,[38] preparations that could not contain the lipid fraction have been shown to produce a true polycythemia Does erythropoietin initiate the erythroblastic process in the reticular cell as well as in the undifferentiated blast? Is erythropoietin necessary not only for initiation of this differentiation in cells but for further differentiation of erythrocytic precursors?

In the transfusion induced polycythemic mouse erythropoiesis has been reduced to zero, as judged by absence of reticulocytes from the peripheral blood and the absence of erythroblasts from the marrow or other hematopoietic tissue The introduction into the polycythemic mouse of exogenous erythropoietin as plasma or a purified extract rapidly restores erythropoiesis to its normal range (fig 1) The new cells appear to be normal To actually study which of the individual cell types is affected by erythropoietin may require tissue culture techniques, with particular attention being given to individual types as well as to their maturation stages

The second important consideration concerns the fate of erythropoietin under normal conditions Is it completely utilized in the process of stimulating erythropoiesis, or, in addition, does loss occur by degradation in the marrow or elsewhere?

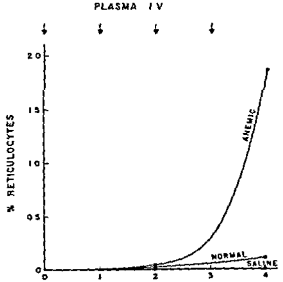

FIG 1 —Assay of anemic plasma in polycythemic mice.

This cannot be answered at present with any degree of certainty because methods of assay do not permit quantitative determination of plasma erythropoietin titer in the range of normal or below Degradation by the liver has been suggested by Jacobsen et al [22] and Prentice and Mirand [37]

We have observed, however, that the rate of disappearance of plasma erythropoietin in rats stimulated by hypoxic hypoxia is the same in normal and in 85 per cent hepatectomized rats This finding would tend to make degradation by the liver a minor factor in the disappearance of erythropoietin

We have observed the plasma erythropoietin titer (a) in rats at altitude (21,500 ft) before nephrectomy, and the fall in titer after nephrectomy while the rats are maintained at altitude, (b) the fall in titer in normal rats upon removal from altitude, (c) the fall in titer in rats following the peak reached after a single injection of cobalt ion The rate of decline in titer is remarkably similar in all these situations These data suggest a plasma erythropoietin half life of about 12 hours

Of considerable interest in this regard are the findings reported previously [26] In this study, bilateral nephrectomy was performed on rats at intervals after the administration of cobalt The rate of disappearance of erythropoietin when the kidneys were excised eight hours after cobalt administration was about the same as that in animals from which the kidneys had not been removed and in which the normal peak titer was reached The rate of disappearance of erythropoietin from the plasma may be a two phase curve, with a rapid initial component and a slower second component (fig 2)

Another group of observations indirectly concerning the problem of utilization of erythropoietin and plasma disappearance rate arises from our experience in the bioassay of plasma specimens from different anemic patients whose hemoglobin was under 8 0 Gm per cent at the time the plasma was obtained *First* not all 'anemic plasma" specimens demonstrate elevated erythropoietin titer In some instances, the heat denatured extract of anemic plasma did not differ from the control, but assay of the whole plasma in the starved rat yielded evidence of an elevated erythropoietin titer It would appear that heat denaturation for 10 minutes is responsible for some destruction However, this destruction is not complete, and, if very high titers of erythropoietin are present, enough hormone survives the heat denaturation to give a positive result When testing an unknown plasma for elevated erythropoietin, our data suggest that it is essential to include at least one group of assay animals that are given whole plasma *Second*, even when whole plasma specimens are tested, many fail to demonstrate an increased titer of erythropoietin The limitations of the assay method have been considered elsewhere [43] It is probable that many of these negative" plasma specimens contain enough erythropoietin to sustain a normal or even increased rate of erythropoiesis, although the sensitivity of the assay procedures employed currently is such that a slight excess of erythropoietin cannot be demonstrated unless large amounts of plasma are obtained and concentrated prior to assay It is in the group of patients whose plasma is negative on bioassay that we anticipated finding individuals in whom erythropoietin will be of clinical importance Clearly, not all patients whose plasma fails to give a positive result on bioassay can be said to be anemic because of a deficiency in erythropoietin *Third*, arising from

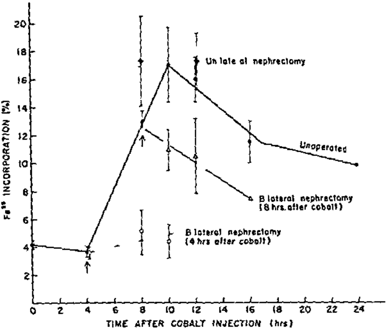

FIG 2 The effect of plasma obtained from rats subjected to nephrectomy and cobalt stimulation on the incorporation of Fe^{59} into the red blood cells of starved recipients Sprague Dawley rats, 350 Gm, were used as donors These were injected with 75 μM of $CoCl_2$ at zero time nephrectomy performed at intervals indicated by arrows and exsanguination at times indicated by points The plasma was assayed in starved rats using per cent Fe^{59} incorporation Each point represents the average per cent of Fe^{59} incorporation into the red blood cells of five rats The standard deviations are given (from Jacobson L O et al [39])

our clinical experience and related to the problem of utilization is the observation that patients with unusually high titers give evidence of reduced erythropoietic activity, as measured by the number of reticulocytes and the morphologic appearance of their marrow Plasmas from patients with aplastic anemia (7 cases), erythrogenesis imperfecta (3 cases), acute leukemia (4 cases), and myelofibrosis, with little to suggest compensatory myeloid metaplasia (1 case), give unusually high assay results, while those from patients with anemias characterized by hemolytic anemia, pernicious anemia, and acute blood loss have generally been characterized by less dramatic elevations, and, in some instances, by no elevation that we could discern This has suggested to us the possibility that erythropoietin is consumed or in some manner degraded by the rapidly proliferating marrow precursors Stohlman and Brecher[42] have also considered this possibility and presented experimental evidence to substantiate this hypothesis *Fourth*, is the clinical observation of a high urinary titer of erythropoietin found in patients whose plasma titer is high A rapid fall in the plasma and urine titers occurs following transfusion The urinary titer falls to a level below which activity can be demonstrated, and constitutes strong support of the concept of the dynamic equilibrium of erythropoiesis operating under the control of erythropoietin It also indicates the delicate reciprocal relationship between erythropoietin production and the capacity of the organism to maintain adequate oxygenation at a cellular level

TABLE 3.—*Plasma and Urinary Erythropoietin Titer Specimens from Patient With Aplastic Anemia Assay in Starved Rat.*

Plasma		Urine	
Volume*	Assay Result† (%)	Volume*	Assay Result† (%)
4 ml	36.0	20 ml	37.0
2 ml	31.0	10 ml	33.3
1 ml	25.6	5 ml	25.7
0.5 ml	17.8	2.5 ml	15.2
0	4.5	0	3.7

* All specimens concentrated or diluted to volume of 4 ml given as 2 daily injections of 2 ml each.

† Incorporation of a tracer dose of Fe^{59} into peripheral red cells.

Finally the significance of urinary excretion of the hormone in its over all economy must be considered. The fact that urinary excretion occurs in animals after phenylhydrazine stimulation[21] and is found in the urine of patients with certain disease states,[17, 27, 46] influences our thinking of the fate of the hormone under normal conditions. Presence of the hormone in the urine of normal individuals has not been reported, but this does not mean that it is not present in small quantity. This matter should be explored thoroughly, since these studies might reveal whether erythropoietin is a threshold substance which it appears to be.

Recently we have demonstrated conclusively in one patient a fivefold relationship between urinary and plasma activity (table 3). On several occasions we have observed a decrease in the plasma and urinary erythropoietin titer of an anemic patient following transfusion. This observation has also been reported by Medici et al.[30]

SITE OF ERYTHROPOIETIN PRODUCTION

Shortly after their original description of the hormone Carnot and Deflandre[3] published a report claiming that the site of formation of the hormone was in the marrow since they could demonstrate erythropoietic activity in aqueous extracts of femoral marrow. According to them, these extracts were as active as serum from anemic animals. They also found appreciable but inconstant activity in extracts of brain.

That a normal bone marrow or rather a normally proliferating blood forming tissue, is not necessary for erythropoietin production has been shown by the findings of Stohlman and co-workers[43] Erslev and Lavietes,[7] and Linman and Bethell.[27] X irradiation and the nitrogen mustards which reduce the marrow and lymphatic tissue to a reticulum stroma, as judged by histologic studies do not impair the capacity of animals to respond to anemic stimuli by increasing the circulating level of erythropoietin. It is possible that an endocrine function, or the formation of some substance by reticulum cells of the marrow, may still function without impairment at a time when the blood forming tissue appears to be inactive with respect to hematopoiesis. The early observation made by Carnot and

Deflandre was not confirmed by Gordon, Piliero, and Tannenbaum,[14] who found no activity in heated extracts of marrow

The possibility that the pituitary might be involved in the formation of erythropoietin,[4] was made unlikely by observation that hypophysectomized rats would respond to bleeding by a reticulocytosis[8] and by an increased titer of erythropoietin [5] [8]

Gordon and his associates[15] concluded that neither liver, spleen, thymus, lung, brain, skeletal muscle, marrow, nor red cells was the source of erythropoietin, since heated extracts of these tissues derived from anemic animals were devoid of activity Alternative conclusions proposed were that there was no significant storage in the organ of formation, or that the hormone was produced as an inactive precursor that was activated in the circulatory system

Jacobson et al ,[26] by extirpating organs before studying the response to stimuli such as cobalt or phlebotomy, found that removal of spleen, stomach, intestines, gonads, adrenals, thymus, pancreas, or 90 per cent of the liver caused no appreciable inhibition of response, while removal of the kidneys completely abolished it (table 4)

In an attempt to control the effect of the uremia resulting from bilateral nephrectomy, the response of rats whose ureters were ligated was studied While the resulting uremias, as measured by blood urea nitrogen, were similar in both the ureter-ligated and the bilaterally-nephrectomized animals, the former did not lose their capacity to respond to cobalt or phlebotomy, while the animals without kidneys had completely lost such responsiveness When the time between ureter ligation and stimulus is prolonged, there does appear to be a diminished responsiveness [12] We feel, however, that this impairment may be due to damage to the kidneys caused by mechanical trauma At autopsy, the kidneys from rats whose ureters were ligated were about twice the normal size The surfaces were pale,

TABLE 4 —*The Effect of Plasma Obtained From Rats Subjected to Organ Excision and Co^{++} Stimulation Upon the Incorporation of Fe^{59} Into the Red Blood Cells of Starved Recipients*

Organ Removed From Donor Prior to Co^{++} Injection*	Stimulus	Assay of Donor Plasma in Starved Rats Using % Fe^{59} Incorporation Response of Recipient to Plasma Preparation
None	None	3 7 (0 4)†
None	Cobalt (250 µM/Kg)	14 4 (1 5)
Adrenals and gonads	Cobalt	15 1 (0 9)
90% of the liver	Cobalt	12 4 (0 4)
Stomach, intestines, spleen, pancreas	Cobalt	11 7 (1 2)
Kidneys	Cobalt	4 5 (0 7)
None	None	4 8 (0 6)
None	Cobalt	16 5‡
Thymus	Cobalt	10 3 (1 6)

* Immediately following the surgical procedure, the rats (Sprague Dawley, 300 Gm) were injected subcutaneously with cobaltous chloride, and 12 hours later the blood was collected via cardiac puncture Plasma thus obtained was assayed in the starved rats by standard procedures

† Standard error

‡ Three rats

and after cutting the kidney perpendicularly to the anterior posterior axis, the cut surfaces were observed to be blanched, especially in the cortical region

Additional evidence that the kidney is involved intimately in erythropoietin formation can be seen in figure 2 This experiment shows the results of nephrectomy at intervals after cobalt administration If the kidneys are removed before there has been an appreciable rise in plasma erythropoietin titer (4 hr), no subsequent rise occurs This is also true when the kidneys are removed after the titer has risen, but before it has reached its maximum value (8 hr)

The rate of disappearance of plasma erythropoietin, when the kidneys are excised eight hours after cobalt administration, is about the same as in animals in which the kidneys are not removed and the normal peak titer is reached The observation that the rates of loss of erythropoietin titer in these two cases are similar would indicate that no toxic reaction is caused by the absence of kidneys which might be responsible for the halt in erythropoietin reproduction

Objections to the conclusion that the kidneys are involved in erythropoiesis have been published Erslev[6] reported data suggesting that it was the uremic state that was responsible for the lack of response of nephrectomized animals since bilaterally nephrectomized and ureter ligated rabbits used in his study did not show an increase in erythropoietin titer after they had been bled On the other hand, Mirand and Prentice[31] reported elevated erythropoietin levels in plasma of bilaterally nephrectomized, as well as ureter ligated, rats subjected to hypoxic hypoxia These latter authors suggested that there were two different erythropoietins formed in response to different stimuli Our own findings from studies of animals subjected to hypoxic hypoxia[12] fully confirmed our earlier observations that ureter ligated animals retained the capacity to respond to bleeding or cobalt by the production of an increased plasma erythropoietin titer about as well as unoperated controls, whereas animals that were bilaterally nephrectomized did not

In addition to assaying plasma samples by the technic of Fe^{59} incorporation we used the reticulocytic response in polycythemic mice[25] as a means of assay and obtained quite similar results i e , plasma from normal and ureter ligated animals after stimulation (anemic anoxia and hypoxic anoxia) had high titers, plasma from normal unstimulated animals had none, and plasma from nephrectomized animals subjected to these stimuli had only a questionable titer (table 5) The finding of any reticulocytes at all in the last group suggests that there may possibly be an extrarenal source of the hormone There is about a tenfold difference in the reticulocyte response of those mice given plasma from normal stimulated animals and of those given plasma from nephrectomized stimulated animals If we can consider these responses to be related quantitatively to the amount of erythropoietin in the plasma samples, we might conclude that any extrarenal source of erythropoietin, if one exists, is a relatively minor one This observation cannot explain the findings of Mirand and Prentice,[31] since they reported that bilaterally nephrectomized rats subjected to hypoxic anoxia attained erythropoietin titers equal to those of normal animals subjected to hypoxic anoxia

One obvious desideratum would be the demonstration of erythropoietin in kidney extracts We have recently been able to show activity in kidney prepara

TABLE 5.—*Effect of Plasma From Normal Unoperated, Nephrectomized and Ureter Ligated Rats Exposed to a Simulated Altitude of 21,500 Feet for 8, 16, or 24 Hours on the Reticulocyte Response of Mice With a Transfusion Induced Polycythemia as Compared With Plasma From Normal and Starved Rats*

	Reticulocytes (%) in Polycythemic Mice							
	Group							
	1		2		3		4	5
Time in Low O₂ (Hr.)	Normal Unoperated	S.D.	Nephrectomy	S.D.	Ureters Ligated	S.D.	Starved	Saline
8	0.9	0.33 (9)	0.18	0.36 (9)	0.7	0.26 (9)	—	—
16	1.71	0.49 (7)	0.11	0.14 (5)	1.38	0.47 (7)	—	—
24	1.78	1.47 (8)	0.00	— (3)	1.26	0.45 (4)	—	—
0 (not exposed)	0.01	0.02 (8)					0.00 (4)	0.00 (14)

S.D. = Standard deviation.
Figures in parenthesis indicate number of animals in each group.

tions, which could not have been due to the presence of plasma in the kidney.[13] Such experiments, however, have not been uniformly successful, and much more definitive evidence is required before the kidney can be accepted unequivocally as the main or sole site of production.

While the resolution of differences in both results and interpretations from the various laboratories awaits further, more decisive experimentation, some indirect evidence concerning the role of the kidney in the elaboration of erythropoietin is worthy of attention. Naets[33, 34] has shown that nephrectomized dogs kept alive by peritoneal dialysis had a severely suppressed rate of erythropoiesis, while ureter ligated dogs underwent only a minimal depression in this regard. Gurney and Pan[17] demonstrated that seven out of seven anemic patients with uremia did not have elevated erythropoietin titers, even though they were as anemic as other patients who did have increased amounts of erythropoietin in their plasma, and similar results were recently reported in 15 of 16 instances where plasma from anemic uremic patients was assayed.[11]

Very recently, Osnes[35] has published some data indicating that in mice, X irradiation of the kidneys with large doses can cause a severe uremia along with anemia. In point of fact, the anemia is apparent before the uremia becomes severe. If part of the kidney is shielded from X irradiation, uremia still results, but anemia does not appear.

PURIFICATION OF ERYTHROPOIETIN

In collaboration with W. F. White of The Research Division, Armour and Company, we have succeeded in the preparation of a non anaphylactic, biologically active sheep erythropoietin. The method of preparation involves three successive ion-exchange steps using DEAE cellulose and XE-97 resin. The product, when assayed in starved rats by the iron incorporation method, contains 1.3 to 5.0 arbitrary units per mg. of protein, as contrasted with 0.007 units per mg. protein in the original anemic sheep plasma. The active fraction is homogeneous by ultracentrifugal analysis at 3 pH values, by moving boundary electrophoresis at 3 pH values, and by chromatography on DEAE cellulose It has the properties of an α_1 glycoprotein and a molecular weight of about 40 000 by light scattering It was found to contain 83 per cent protein, 6 5 per cent carbohydrate, and 16 per cent sialic acid In spite of its apparent homogeneity, we have been able to fractionate it further into a fraction containing most of the α_1 glycoprotein and none of the activity, and one containing very little protein and most of the activity This last fraction has an activity of about 150 units per mg protein, and contains 55 per cent protein, 11 per cent carbohydrate, and 6 per cent sialic acid We have not yet accumulated enough to study its physical properties and homogeneity It is clear that extreme caution must be exercised in the interpretation of the properties of such fractions with regard to absolute purity

SUMMARY

Some of the factors that control erythropoiesis through the control of the production of erythropoietin have been studied A hypothesis that the steady state of red cell formation is controlled by the relationship of the tissue demand for oxygen to the supply of oxygen available, rather than by either factor alone, has been presented with experimental data to substantiate it

High titers of erythropoietin found in plasma and urine of severely anemic patients whose marrow shows reduced erythropoietic activity suggests that erythropoietin may be consumed or degraded by actively proliferating erythrocytic elements

The site of erythropoietin production has been studied by stimulating animals with either cobalt, hemorrhage, or hypoxia after extirpation of various organs The only organs whose removal abolished the response to these stimuli were the kidneys Animals with uremia comparable to that of nephrectomized animals were prepared by bilateral ureter ligation and found to respond to the above stimuli about as well as normal animals, indicating that it is not the uremia itself that interferes with erythropoietin formation The disappearance of erythropoietin from the circulation of nephrectomized animals follows the same time course as that seen in normal animals The data suggest very strongly that the kidney is the site of erythropoietin formation or at least is very greatly involved in its production

The isolation of erythropoietin from anemic sheep plasma has progressed far enough to yield a product suitable for clinical testing The purified material is non antigenic for guinea pigs and is homogeneous by electrophoresis column chromatography, and ultracentrifugal analysis While the data are insufficient for a definitive statement about the absolute purity of this preparation we suggest that it is not in a final state of purification

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On the Mucoprotein Erythropoietic Factor

By Henry Borsook

THIS DISCUSSION will be limited to the erythropoietic substance that appears to be a mucoprotein Linman and Bethell have reported extracting a nonprotein erythropoietic factor from the blood of anemic rabbits The mucoprotein factor has been found in the blood of rabbits[1, 1a, 2] and dogs[3] made severely anemic with phenylhydrazine, in the urine of human patients with Cooley's anemia[4] and aplastic anemia,[5] and in the goat after severe and prolonged bleeding[6] Dr Keighley's findings in our laboratory indicate that to obtain a potent plasma the anemia must be so severe as to render the animal nearly moribund, the blood drawn as soon as possible after the animal s temperature has dropped sharply In Gordon's cases of Cooley's anemia, the erythropoietic potency of the urine was greater the more severe the anemia In Van Dyke's case of aplastic anemia,[5] whose urine was highly erythropoietic, the anemia was severe

Erythropoietic potency (of the mucoprotein factor) is assayed currently by three different methods reticulocytosis in mice, increased uptake of Fe^{59} in rats, and polycythemia in adult rats at normal altitude In the first two tests the material is injected subcutaneously once daily for three days, and the test animal is examined the next day In the polycythemia test the material is injected subcutaneously daily for two weeks, usually the response is only slight after the first week

The plasma of a rabbit made severely anemic with phenylhydrazine gives a positive response in the two short periods (three-day tests), but instead of a polycythemia, it causes a drop in hemoglobin and red cell count in the longer (two weeks) test On the other hand, after the plasma has been boiled for a few minutes at pH 5 5, the filtrate (plasma filtrate) gives positive responses by all three tests

Keighley has found that the anemic rabbit plasma is antigenic in the rat, and hence provokes the formation of antibodies, whereas the plasma filtrate although it contains a number of proteins, is not antigenic[7] Antigenicity of the proteins appears to be associated with a hemolytic effect in the two week test Whether the boiling abolished their antigenicity or whether the proteins that remain in solution after boiling are not antigenic even before boiling has not been determined When a dialyzed (salt free) fraction of the plasma containing the active erythropoietic protein (supernatant of half saturated ammonium sulfate) is boiled, little or no activity was found in the filtrate Evidently, salt is required for retention of the activity in solution after boiling

The erythropoietic protein is not species specific Active rabbit plasma filtrate gave positive responses in the mouse and the rat, and that from the dog gave a positive response in the rat. The active material from human urine (Cooley's

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anemia) was active in the rat and from a case of aplastic anemia was found to be active in the rat and monkey

Van Dyke states that the mucoprotein from the urine of his aplastic anemia case which is highly active erythropoietically in the rat and monkey when injected into guinea pigs stimulates granulocyte production and has no erythropoietic action These observations were made by Yoffey Keighley has confirmed these observations with active material obtained from rabbit anemic plasma filtrate It is possible that the proteins of rabbit plasma filtrate and of human urine are antigenic in the guinea pig

Lowy in our laboratory has been engaged in the purification of the active material Fractional alcohol precipitation dialysis followed by chromatography on cellulose anion and cation exchange columns has yielded material that meets the criteria for a pure protein with respect to the shape of the eluted bands in column chromatography in the ultracentrifuge and by electrophoresis It is a mucoprotein i e it contains sialic acid and carbohydrates It is stable to boiling at pH 5 5 in isotonic saline and is not dialyzable A tentative estimate of its molecular weight is 29 000 We have reason to think that erythropoietic protein of approximately this degree of purity has been obtained in other laboratories (Jacobson Rambach)

Yet one cannot without reservations at present accept this material as pure because a fraction with somewhat similar properties is obtained from normal rabbit urine which gives no erythropoietic response at a dose (in terms of protein) 30 times that of the fraction from anemic plasma which gave a maximal response There are two possibilities that the active material is only a small fraction of the apparently pure protein obtained from anemic plasma filtrate and that it is so similar chemically to the inactive protein which constitutes the bulk of this fraction that the tests so far employed do not detect the presence of more than one protein in this fraction The other alternative possibility is that the active fraction obtained from anemic plasma filtrate is the major (if not the sole) component of that fraction and that the inactive material obtained from normal plasma filtrate will be found to be different Chromatographically the plasma filtrates of anemic and normal plasma are different the peak corresponding to the active material is relatively much higher in the anemic than in the normal and an adjoining inactive peak emerging earlier on elution with NaCl from a DEAE column is relatively high in inactive plasma filtrate and almost absent from anemic plasma Of course there must be chemical and physical differences between the active and inactive proteins We are now trying to identify such differences

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Discussion of Identity of Erythropoietin and Its Site of Production

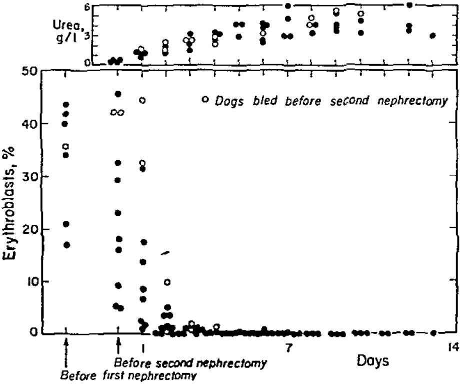
J W Linman Our studies indicate that there are two humoral erythropoietic factors A relatively thermolabile ether insoluble agent which enhances hemoglobin production is demonstrable in active plasmas tested in the unmodified state or after boiling for only five to ten minutes Although the ether soluble fractions of these same plasmas or extracts thereof which have been processed by boiling for 30 or more minutes fail to augment hemoglobin synthesis in recipient animals they do induce in normal rats a singular type of erythropoietic response The latter is characterized by erythrocytosis due to the production of microcytes with decreased osmotic resistance, reticulocytosis and a three to fourfold increase in the number of nucleated erythrocytic elements in the marrow This response is not associated with increases in hemoglobin hematocrit or Fe^{59} uptake, and we believe it is the result of accelerated proliferative or mitotic activity of erythrocytic precursors in the absence of a comparable stimulus to hemoglobin synthesis

On the basis of currently available data we concur with the theory that the humoral factor which augments hemoglobin production and appears to be a mucoprotein most likely increases erythrocytic differentiation of the multipotential myeloid reticulum cells and that subsequent maturation and hemoglobin synthesis probably occur simultaneously and at a fixed rate It is our opinion however, that the number of homeoplastic cellular divisions which the myeloid erythrocytic precursors undergo during maturation is governed by a second humoral factor, which is thermostable ether soluble and probably a lipid Individually these two agents would appear to control respectively the quantity of hemoglobin and number of erythrocytes produced with their combined activities determining in addition the size and hemoglobin content of each red cell

We have yet to study an active plasma regardless of the experimental or clinical conditions under which it was obtained that has not possessed erythropoietic stimulatory activity attributable to both humoral factors Since their combined effect results in the production of increased numbers of normal cells the response in recipient animals to active whole plasmas or extracts which have been prepared by boiling for only short periods of time is characterized by an increase in all erythroid determinations However the differences in the chemical and physiologic properties of these two factors are such that they may explain some of the apparent discrepancies in experimental observations which have been described

J P Naets For the past two years we have studied erythropoiesis in the nephrectomized dog[1] The dogs were nephrectomized in two steps the second kidney being removed two to four weeks after the first Thereafter the dogs were kept alive eight to fourteen days by peritoneal dialysis

The percentage of erythroblasts in the marrow is shown in Discussion figure 1 Each point represents the percentage of erythroblasts in one animal The value prior to nephrectomy is given on the left Two days after nephrectomy there is a striking decrease in erythroblasts and after three days there are no more than three to



DISCUSSION FIG 1 —Depletion of dog bone marrow erythroblasts after bilateral nephrectomy

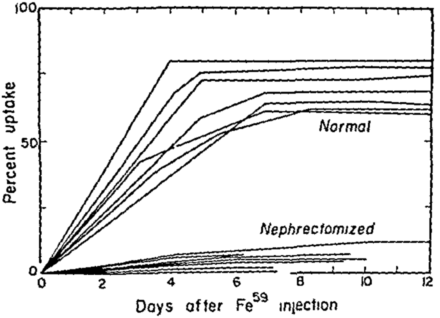
four erythroblasts per 1000 nucleated cells After four days only an occasional erythroblast is seen After the fifth day not a single erythroblast was seen in 12 dogs The urea levels are given in the top of the chart The urea reached a level of about 3 Gm /L

Three days after nephrectomy the dogs were injected with Fe^{59}, and the iron turnover and uptake measured The $T_{1/2}$ for plasma Fe^{59} clearance of the normal group averaged 93 minutes, and in the nephrectomized ones 300 minutes The uptake of Fe^{59} in the red cells is very low in the nephrectomized dogs, averaging five per cent in contrast to 70 per cent of the injected dose in the control dogs (Discussion fig 2) The total iron turnover in the nephrectomized group was 0 25 mg /kg /day, and in the normal, 0 62 (Discussion fig 3) A substantial amount of the Fe^{59} went to the liver in the nephrectomized dog, in contrast to the normal, where it went mainly to erythrocytes

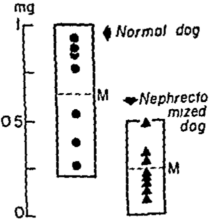
Dogs with bilateral ligation of the ureter were almost completely normal despite the same degree of urea retention and the same degree of starvation (a dog after ureter ligation or nephrectomy, doesn t eat) The disposition of Fe^{59} in normal nephrectomized and bilateral ureteral ligation is shown in Discussion figure 4 The Fe^{59} uptake into the red cells in nephrectomized and ligated ureter dogs is compared in Discussion figure 5

Finally, we injected a very active erythropoietic extract (kindly supplied by Dr Van Dyke from the urine of a patient with aplastic anemia) into nephrectomized dogs and we obtained the erythropoietic response shown in Discussion figure 6 The dog was injected on each of two days with 150 mg of extract There was an acute reticulocyte response in the normal dog The same dog when injected with this factor after a bilateral nephrectomy had a substantial increase in erythroblasts at a time when usually none are seen in the nephrectomized animal

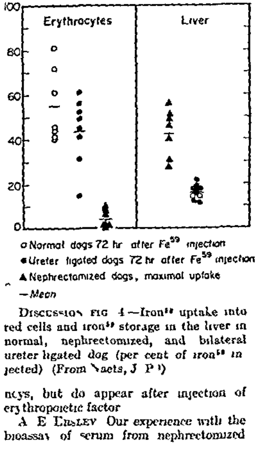
Thus it is evident that the toxic products in the blood of uremic animals does not impair production of red cells From this experience we conclude (1) that bilateral



DISCUSSION FIG 2.—Iron^{59} uptake into red cells in normal dogs and after bilateral nephrectomy (From Naets, J. P.[1])



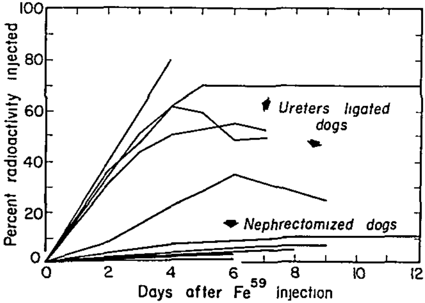
DISCUSSION FIG 3.—Iron turnover (mg/kg/day) in normal and bilateral nephrectomized dogs



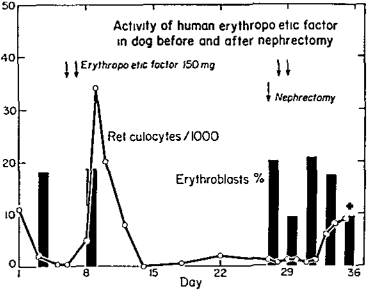
DISCUSSION FIG 4.—Iron^{59} uptake into red cells and iron^{59} storage in the liver in normal, nephrectomized, and bilateral ureter ligated dog (per cent of iron^{59} injected) (From Naets, J. P.[1])

nephrectomy in the dog reduces erythropoiesis, and (2) that the retention of toxic products in the dog after bilateral ureteral ligation doesn't impair the marrow, at least for a period of eight or ten days. Perhaps the erythropoietic factor is made in the kidneys, and acts by inducing differentiation of undifferentiated (stem) cells to erythroblasts. This is supported by the observation that erythroblasts are no longer seen in the marrow of dogs without kidneys, but do appear after injection of erythropoietic factor.

A. E. ERSLEV: Our experience with the bioassay of serum from nephrectomized



Discussion fig. 5.—Iron^{59} uptake into red cells in the dog after bilateral nephrectomy and after bilateral ureter ligation (From Naets, J. P.[1])



Discussion fig. 6.—Effect of human urinary erythropoietic factor on reticulocytes and bone marrow erythroblasts in a dog before and after bilateral nephrectomy

and normal animals along with the discrepancy which exists between the work carried out by Mirand and Prentice[2] and by Jacobson and co-workers[3] have taught us that the technics currently used in measuring erythropoietic factor are exceedingly crude. The technics are reliable only when a large concentration of erythropoietic factor is present but they cannot be used to demonstrate small amounts. Consequently we decided to evaluate red cell production rather than production of erythropoietic factor in the nephrectomized animal itself. Our results in rabbits are completely different from the observations in dogs made by Dr Naets Red cell production was severely suppressed in nephrectomized uremic rabbits but some degree of erythropoietic activity was present up to nine days after nephrectomy

I have great trouble in believing that uremic ureter ligated animals can behave like normal animals and have a normal rate of red cell production and a normal bone marrow We know that red cell production is exceedingly sensitive to small infections inflammations or any condition which changes the metabolic set-up Is there anything as powerful as urinary retention even when produced by bilateral ureter ligation in changing the metabolic environment? Our data indicate that the red cell production and the response to an anoxic stimulus is suppressed right after the onset of uremia whether it is induced by nephrectomy or by ureter ligation

E A MIRAND We support in part Dr Jacobson s concept that the kidney is the site of production of erythropoietin. Extensive experiments using nephrectomized normal and hypophysectomized rats demonstrate the capacity of the nephrectomized animal to produce erythropoietin in response to hypoxic anoxia but not in response to bleeding or cobalt administration[3, 4] There have been a few instances when young nephrectomized normal or hypophysectomized rats did lose the capacity to respond to hypoxic anoxia. Studies are in progress to explain this phenomenon Recently Dr Piliero and his associates[4] have confirmed the capacity of nephrectomized rats to respond to hypoxic anoxia with an increased production of erythropoietin These findings do not lend support to the kidney being the sole site of production of erythropoietin Whether erythropoietin is produced in response to hypoxic anoxia is still unknown

J P NAETS I was unable to find erythropoietin in the plasma of one dog without a kidney despite the fact that the hematocrit was less than 15 per cent

I don t agree with the remarks about clinical uremia I have been impressed by the fact that some anuric patients with severe uremia had very few erythroblasts in the marrow It is known that patients with chronic azotemia for six months or more do not have such marked changes in the marrow Some anuric patients showed a substantial decrease in Fe^{59} uptake and in this respect these patients are just like nephrectomized dogs[5] I have never seen this in chronic uremic patients. Sometimes uremic patients have a very normal uptake of Fe^{59} sometimes lower, but they never reach the low level of anuric patients

F H GARDNER Is it my understanding Dr Naets that some of the uremic patients do have a normal uptake?

J P NAETS Yes

F H. GARDNER I think Dr Erslev knows of one patient studied last year who had a bilateral nephrectomy and before receiving extensive whole body radiation had an iron uptake for four days of 30 per cent Thus we would have to assume that the human had the ability to go ahead and incorporate iron into hemoglobin to that extent

M. BESSIS Dr Richet and collaborators[6] in 1954 studied 21 patients with acute renal failure treated by dialysis on the artificial kidney They observed the total disappearance of erythroblasts in bone marrow starting about the fifth day The same thing was not observed in chronic nephritis. This phenomenon observed in humans confirms a relationship between the kidneys and hematopoiesis

J M YOFFEY We have accumulated a tremendous mass of quantitative data on the guinea pig It is a miserable animal in many ways It has no tail and it has no decent veins for intravenous injection Dr Van Dyke gave us a concentrate of his active erythropoietin from urine which in rats has produced polycythemia and reticulocytosis. Drs D G Osmond and P J Roylance gave it to guinea pigs In the marrow we observed not erythropoietic, but granulopoietic stimulation. Dr Van Dyke then found that by his Fe^{59} method this active concentrate did not show any erythropoietic activity in the *guinea pig* When we tried the concentrate in rats we too got polycythemia We don t quite know whether one and the same substance can produce marked granulopoietic stimulation in one animal, and in another, erythropoietic stimulation or whether there is some other factor operative

H BORSOOK The material which Dr Van Dyke was kind enough to send us from urine appears to be different It is far less soluble than the material we obtain from blood I am not sure yet whether the differ ence represents an impurity The active material may be the same

D C Van Dyke Dr Gordon has presented evidence that red cells produced under the influence of erythropoietin can not be distinguished from normal red cells by the criteria he used I would like to mention one observation that suggests that the red cells produced under the stimulation of erythropoietin may have a shortened life span An adult male Java monkey was given 75 mg of human urinary erythropoietin daily for 16 days On the tenth day the circulating red cell mass had increased by 30 per cent, and two days after the last injection by 61 per cent The circulating red cell mass had returned to the pretreatment level 19 days after the last injection It has been shown that following acute bleeding there is a rapid rise and fall in the glycine 2-C^{14} specific activity curve which Berlin et al interpret as indicating the production of a short lived erythrocyte[7] We put the same monkey in an altitude chamber at a simulated altitude of 18 000 feet for 16 days Two weeks later the total circulating red cell volume was found to be increased by 97 per cent Nineteen days after removal from the altitude chamber, the circulating red cell volume was found to have been maintained at 95 per cent above the pretreatment level

A Erslev Did the hemoglobin and red counts increase by 60 per cent?

D C Van Dyke After treatment with erythropoietin, when the red cell volume had increased by 61 per cent, the hematocrit increased from 36 to 45 and the hemoglobin from 9 to 12 Gm per cent After exposure to hypoxia, when the red cell volume had increased by 97 per cent, the hematocrit increased from 38 8 to 52 9 and the hemoglobin from 10 0 to 13 6 Gm per cent

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Section VI. Mathematical Considerations

J. Cornfield, *Chairman*

Introduction

By JEROME CORNFIELD

MY FUNCTION is to set the ground for the two main papers of the afternoon. I think I can perhaps best do this by considering a hypothetical 19th century hematologist who has somehow been transported through time to this conference. He finds the new labeling technics and the results obtained with them of great interest. I think he might be a little puzzled, however, by the present session and might wonder how hematology had gotten mixed up with mathematics. There are a number of answers that might be given, but I shall confine myself to one, namely, that in many situations it is not possible to interpret observations until one has written down in a certain amount of detail exactly what one thinks has happened to produce them. I shall illustrate this by considering the estimation of the average length of sojourn of label in the circulation.

Much of the data presented at the conference seem to take the following form. If we consider the total amount of label appearing in the circulation (it is simpler to talk about total amount of label than about number of labeled cells) we see it enter, starting at zero level, and increase to a peak, and then return to the initial level. Such a curve leads the mathematician to conclude that two things are going on simultaneously. Label enters and label leaves; both of these things are going on at the same time, and what one sees is the resultant. We wish to estimate the average sojourn of a labeled cell in the circulation from observations of this type, but before we can do so we must specify in just a little bit more detail what is happening.

We can start by assuming that the label is picked up some place by newly formed cellular elements, and that the process of uptake is confined to a very short period of time. We can imagine looking at any one cell which has just taken up label and determining the interval that elapses between the time of uptake and the time at which it appeared in the peripheral circulation. We denote the length of this interval by t. t will vary from cell to cell, but we can imagine a frequency distribution which shows the distribution of label by time elapsing between its uptake and appearance in the peripheral circulation.

We denote the amount of label appearing in the infinitesimal interval t to $t + dt$ by $f_1(t)dt$. If we denote the interval elapsing until the appearance of the last atom of label to appear in the circulation by I_1 and the average length of the

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interval elapsing between uptake and appearance by t_1 then

$$t_1 = \int_0^{L_1} tf_1(t)\,dt \Big/ \int_0^{L_1} f_1(t)\,dt. \tag{1}$$

We can imagine another set of observations on each cell, namely, the interval elapsing between uptake of label and the disappearance of the label from the circulation. Denote this interval by t_2, an upper limit to it by L_2, the amount lost in the interval t to $t + dt$ by $f_2(t)dt$, and the average length of the interval elapsing between uptake and disappearance by t_2. Then

$$t_2 = \int_0^{L_2} tf_2(t)\,dt \Big/ \int_0^{L_2} f_2(t)\,dt \tag{2}$$

The quantity of interest, the average sojourn, is then simply $t_2 - t_1$. But we have no direct observations on $t_2 - t_1$. All we have are observations on amount of label present at any time. This is easily seen to be

$$\begin{array}{l}\text{Observed amount of}\\ \text{label at time T}\end{array} = \int_0^{T} f_1(t)\,dt - \int_0^{T} f_2(t)\,dt \tag{3}$$

The formal question to be considered, then, is whether average sojourn can be estimated from the observed amount of label in the circulation at various times. If the amount of label present at any time is an observable quantity, so then is its change between any two times. Thus,

$$\begin{array}{l}\text{Change in amount of}\\ \text{label between time}\\ \text{and } t + \text{dt}\end{array} = f_1(t)\,dt - f_2(t)\,dt \tag{4}$$

is an observable quantity and so is

$$\int_0^{L_2} t[f_1(t) - f_2(t)]dt \tag{5}$$

If we assume that the total amount of label disappearing is equal to the total amount entering, we may write

$$\int_0^{L_1} f_1(t)dt = \int_0^{L_2} f_2(t)dt \tag{6}$$

and

$$t_2 - t_1 = \frac{\displaystyle\int_0^{L_2} t[f_2(t) - f_1(t)]dt}{\displaystyle\int_0^{L_1} f_1(t)dt} \tag{7}$$

The numerator of this quantity is given by (5) and hence is observable. If the denominator is also observable, then average sojourn can be estimated and if it is not observable it cannot be estimated.

Because of (6) there is at least one value of t say t_0, for which

$$(8) \qquad f_1(t) \begin{matrix} > \\ = \\ < \end{matrix} f_2(t) \qquad \begin{array}{c} \text{depending on} \\ \text{whether} \\ t < t_0 \\ t = t_0 \\ t > t_0 \end{array}$$

and the data presented at this conference suggest there is only one such value of t t_0 is the time at which the amount disappearing is equal to the amount appearing Clearly

$$(9) \qquad t_0 \leq L_1$$

since for any $t > L_1$, $f_1(t) = 0$ Furthermore from (4) t_0 is an observable quantity Hence one can compute

$$(10) \qquad \text{Amount of label present at time } t_0 = \int_0^{t_0} f_1(t)dt - \int_0^{t_0} f_2(t)dt$$

Now the expression in (10) is less than or equal to the denominator of (7) It will be equal to it when $t_0 = L_1$ and $\int_0^{L_1} f_2(t)dt = 0$ namely, when the last atom of label to arrive in the circulation has appeared before any disappearance has begun, and will be less than it otherwise Briefly then even with the oversimplified assumptions that have been made average sojourn can be estimated only when the frequency distributions $f_1(t)$ and $f_2(t)$ do not overlap Otherwise only an upper limit to this sojourn can be estimated [by substituting (10) in the denominator of (7)] Ordinarily, if the observed amount of label as given by (3), has a considerable plateau this suggests that the two distributions have not overlapped, and that the period of the plateau is one during which label is neither appearing nor disappearing Contrariwise the absence of a plateau suggests the existence of overlap and the nonexistence of conditions permitting an estimate of average sojourn

The moral for the 19th century hematologist, then, is that if one is interested in even so elementary a measurement as average sojourn, it takes a certain amount of nontrivial mathematical analysis to establish whether the conditions for its estimation exist

A Mathematical Model for Multiplication by Binary Fission

By T E Harris

IT MIGHT be desirable to present here a survey of all the mathematical methods that have been useful, or at least popular, in dealing with the growth or decrease of biologic populations However, even if the author knew all the methods that are likely to be appropriate, the brief descriptions that could be given within the limits of this paper would not make in the aggregate a very intelligible account Accordingly, this paper will be limited to a discussion of a mathematical model for the increase of a population of organisms by binary fission The assumptions made in constructing the model are undoubtedly too simple, but it will be indicated how similar methods can be used to deal with more complex assumptions, with the hope that the methods can also be applied to problems of interest to this Conference

The growth of a biologic population is an example of a random (or stochastic) process, that is, a process developing over a period of time, whose development is subject to random fluctuations Given a biologic random process, which is in general quite complex, the problem is to pick a mathematical random process that will imitate it as nearly as possible and still be amenable to mathematical analysis

The simplest types of random processes are the *Markov processes* there is a well-developed set of technics, involving differential or difference equations, for dealing with them A good introduction to the theory and application of these processes is given in the text of Feller[8], there is no point in trying to cover the same ground here In what follows, a knowledge of these processes is not assumed

This paper will describe a mathematical random process of the type that has been called *regenerative* Such processes are somewhat more general than Markov processes, and some of them possess features that make their treatment almost as simple They seem particularly adapted to certain problems of population growth An investigator with some knowledge of Markov processes and regenerative processes has an effective set of mathematical methods at his disposal

The chapters of this paper are designated by Roman numerals Formulas are numbered consecutively throughout each chapter, with those in the same chapter referred to by number, such as (4), and designations such as (II 4) used for formulas in a different chapter Similarly, reference is made to Section 1 or Section II 1

No attempt has been made to provide a complete bibliography or to give the history of the various mathematical results However, there are several reference works given, some of these contain lengthy bibliographies

Throughout the paper, the symbol P is reserved for probability" (of the following event), and E is reserved for "expected value"

From the Rand Corporation, Santa Monica, Calif

I. Some Basic Notions in the Theory of Probability

Some basic ideas and methods in the theory of probability are reviewed.

1. Random variables and moments. It is assumed that the concept of a random variable, X, is known.

We shall consider two cases. (a) The possible values of X are the integers $0, 1, 2, \cdots$. In this case (the *discrete* case) the probability distribution of X is specified by probabilities $p_0, p_1, \cdots$, where $p_k = P(X = k)$ for any integer k. (b) X has a continuous range of values (the *continuous* case), and its distribution is specified by a *density function* $f(x)$, where $f(x)dx = P\{x < X < x + dx\}$. The *cumulative distribution* is $F(x) = \int_{-\infty}^{x} f(t)dt$.

The first moment, or *expected value* of X, designated by EX is $p_1 + 2p_2 + 3p_3 + \cdots = \sum_{k=0}^{\infty} k p_k$ (or $\int_{-\infty}^{\infty} xf(x)dx$ in the continuous case). The second moment is $EX^2 = \sum k^2 p_k$ (or $\int x^2 f(x)dx$); the variance $\sigma^2(x)$ is $EX^2 - (EX)^2$, etc.

2. Generating functions. In the discrete case a very useful role is played by the *generating function* (gf). Suppose X corresponds to certain probabilities $p_0, p_1, \cdots$. The gf of X is defined to be the function of the variable s given by

$$g(s) = p_0 + p_1 s + p_2 s^2 + \cdots. \tag{1}$$

We notice the simple but important fact that $g(1) = p_0 + p_1 + p_2 + \cdots = 1$.

Example. X is the number of heads in 10 tosses of a fair coin. Then

$$p_0 = \binom{10}{0}(\tfrac{1}{2})^{10},\ p_1 = \binom{10}{1}(\tfrac{1}{2})^{10},\ \cdots,$$

where $\binom{10}{k}$ is the binomial coefficient $10!/k!(10 - k)!$. The gf in this case is

$$g(s) = \binom{10}{0}(\tfrac{1}{2})^{10} + \binom{10}{1}(\tfrac{1}{2})^{10} s + \binom{10}{2}(\tfrac{1}{2})^{10} s^2 + \cdots + \binom{10}{10}(\tfrac{1}{2})^{10} s^{10} = (\tfrac{1}{2} + \tfrac{1}{2} s)^{10}. \tag{2}$$

3. Uses of generating functions. We shall find the gf useful in two ways.

(a) *To obtain moments.* If we differentiate $g(s)$ as given by (1) we obtain $g'(s) = p_1 + 2p_2 s + \cdots$. Putting $s = 1$ gives

$$g'(1) = p_1 + 2p_2 + 3p_3 + \cdots = EX. \tag{3}$$

Similarly we see with a little reduction, that

$$g''(1) = 2\cdot 1\, p_2 + 3\cdot 2\, p_3 + 4\cdot 3\, p_4 + \cdots = EX^2 - EX. \tag{4}$$

For example, if $g(s)$ has the form given by (2), then $g'(1) = 10(\tfrac{1}{2} + \tfrac{1}{2})^9(\tfrac{1}{2}) = 5$ and $g''(1) = 10\cdot 9\,(\tfrac{1}{2} + \tfrac{1}{2})^8(\tfrac{1}{2})^2 = 22.5$ and thus $EX = 5$ and $\sigma^2(x) = g''(1) + g'(1) - [g'(1)]^2 = 2.5$. Hence, if we know $g(s)$, we can find the moments of X sometimes more simply than by a direct calculation.

(b) *To add independent random variables.* Suppose X and Y are independent discrete random variables, X having the gf $g_1(s) = p_0 + p_1 s + \cdots$, and Y having the gf $g_2(s) = q_0 + q_1 s + \cdots$. We can verify that the random variable $Z = X + Y$ has the probabilities

$$\begin{aligned} P\{Z = 0\} &= p_0 q_0,\ P\{Z = 1\} = p_0 q_1 + p_1 q_0, \\ P\{Z = k\} &= p_0 q_k + p_1 q_{k-1} + \cdots + p_k q_0, \end{aligned} \tag{5}$$

the last expression in (5) representing the sum of the probabilities of possible ways in which X and Y can add up to k The gf of Z is thus

$$g_3(s) = p_0q_0 + (p_0q_1 + p_1q_0)s + \qquad = g_1(s)g_2(s) \tag{6}$$

and we thus have the important result that *the gf of the sum of two independent random variables X and Y is the product of the gf of X and the gf of Y*

For example, suppose X is the number of heads in ten tosses as above, and Y is the number of heads in a second set of ten tosses Then the gf of $X + Y$ (which is the number of heads in 20 tosses) is

$$(\tfrac{1}{2} + \tfrac{1}{2}s)^{10}(\tfrac{1}{2} + \tfrac{1}{2}s)^{10} = (\tfrac{1}{2} + \tfrac{1}{2}s)^{20}$$

4 Random processes We shall give an informal definition of this term which should be adequate for our needs Suppose that the outcome of an experiment or a sampling procedure is not a single number X but a function $X(t)$ defined for every value of t in some interval We call $X(t)$ a *random function* or *random process* For example $X(t)$ might be the complete weight vs time curve of an animal selected at random from some population of animals, or the plot of the number of bacteria in a culture as a function of time In the latter case, we may think of the culture as having been selected at random from a population of possible cultures

II A Mathematical Model for Multiplication by Binary Fission

1 Basic assumptions We assume that a simple organism (which we shall call a *cell*) has a random lifelength T, with a probability density $f(t)$ At the end of its life it splits into two new cells, each of which divides after a lifelength* that has the same density $f(t)$, and so on We make the basic assumption that there is no correlation or any kind of dependence between the lifelength of one cell and that of another, e g , between mother and daughter or between sisters We shall see later how this assumption can be relaxed

Let us note that if a cell has survived until age t, then the probability that the cell splits during the small time interval $(t,t + dt)$ is $f(t)dt/[1 - F(t)]$, and the function $f(t)/[1 - F(t)]$ is the *age specific death rate* In the special case where $f(t) = ce^{-ct}$, the age specific death rate is $ce^{-ct}/e^{-ct} = c$, and this is the only case where the rate is independent of age We shall see later that the mathematical treatment of this case is particularly simple

2 Derivation of equations for the probabilities Let us first make some definitions

Definitions Let $N(t)$ be the number of organisms living at time t Let $f(t)$, as above, be the probability density for the lifelength of one cell The cumulative distribution is $F(t) = \int_0^t f(u)du$, the lower limit of the integral being 0, because the lifelength is never negative Let $p_k(t) = P[N(t) = k]$ be the probability that there are k cells present at time t, if just one newly born cell (the ancestor) was present at time 0 For simplicity we have not admitted the possibility that a cell dies without issue (similar methods can be used to handle this broader assumption) and so $p_0(t) = 0$

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* The term lifelength as used here, is equivalent to the generation time, i e , it is the interval between two divisions It does not imply death of cell at the termination of life but rather a change of state

We shall make repeated use of the generating function of $N(t)$ defined by

$$G(s,t) = p_1(t)s + p_2(t)s^2 + p_3(t)s^3 +$$

Let us now consider the probabilities $p_1(t)$, $p_2(t)$, etc We can easily evaluate $p_1(t)$ For the only way it can happen that just one cell is present at time t is that the ancestor survives until t Thus

$$p_1(t) = P(T > t) = 1 - F(t) \tag{1}$$

We can find $p_2(t)$ with just a little more effort The only way in which just two cells can be present at t is that the ancestor splits at some time u prior to t and each of the two new families thus created has exactly one member after a further time $t - u$ The probability density for splitting of the ancestor at u is $f(u)$, and the probability that both the new families have one member after a further time $t - u$ is $[p_1(t - u)]^2$ Thus we have

$$p_2(t) = \int_0^t [p_1(t - u)]^2 f(u)du \tag{2}$$

Since we already have obtained $p_1(t)$ from (1), we can now get $p_2(t)$ by integration

It is possible in principle to continue in this way and obtain all the probabilities successively However, the operation gets more complicated at each stage Fortunately we can very easily get an equation for the generating function $G(s,t)$

3 *An equation for the generating function* Let t be some point in time, and let us suppose again that the ancestor splits at a time u prior to t, the probability density for this being $f(u)$ Since we now have two new families that develop independently of one another, the cells present at time t (i e , after a further time $t - u$) will consist of a sum $N_1(t - u) + N_2(t - u)$, where N_1 and N_2 are independent random variables, each of which has the generating function $G(s,t - u)$ Hence the generating function for $N_1(t - u) + N_2(t - u)$ will be $[G(s,t - u)]^2$

Next, suppose the ancestor does not split until after the time t, the probability of this being $1 - F(t)$ In this case there is exactly one cell present at t, and the generating function is just s

Summing up over all possibilities, we see that the generating function $G(s,t)$ must satisfy the equation

$$G(s,t) = \int_0^t G^2(s,t - u)f(u)du + s[1 - F(t)] \tag{3}$$

Equation (3) is an integral equation that cannot be solved explicitly except if $f(u)$ has the very special form ce^{-cu}, where c is a positive constant However, we shall see that a substantial amount of information can be obtained from it

4 *The point of regeneration* Before going farther, we should consider the general principle used in obtaining equation (3), since it is useful in many applications of probability theory The instant when the first cell is replaced by two new ones is a very special moment *if we are interested in what happens after that*

moment we gain no further information by learning more about the past history of the process, because of our assumption that there is no dependence between the life lengths of mother and daughter cells On the other hand, a completely arbitrary moment of time does not have this desirable property For example, suppose we know that 5 cells are present at some particular instant of time The behavior of these cells in the next few moments depends on their ages, which we do not know Accordingly, we would be better off if we knew the complete past history of the process, since this would tell us the ages of the cells present, and condition our estimate of the future The name "point of regeneration" is sometimes used to describe an event having the following property the history of the process before the event has no influence on its behavior after the event occurs Each such event may be said to "regenerate" the process, and hence the name

III STUDY OF THE PROPERTIES OF THE MATHEMATICAL MODEL

1 Derivation of the "renewal equation" Let us first see how equation (II 3) can be used to study $EN(t)$, the expected value of the number of cells existing at time t, and the standard deviation of $N(t)$

Definitions Let $m(t)$ and $\sigma(t)$ be the mean and standard deviation of $N(t)$

We recall from Chapter I that the first moment $m(t)$ is the derivative of $G(s,t)$ with respect to s evaluated at $s = 1$ If we differentiate (II 3) with respect to s we obtain

$$G'(s,t) = \int_0^t 2G(s,t-u)G'(s,t-u)f(u)du + 1 - F(t) \tag{1}$$

Since $G(1,t-u) = 1$ (because G is a generating function) and since $G'(1,t) = EN(t) = m(t)$, we obtain, by putting $s = 1$ in (1), the relation

$$m(t) = 2\int_0^t m(t-u)f(u)du + 1 - F(t) \tag{2}$$

Equation (2) is simpler to investigate than equation (II 3), being an example of the so-called "integral equation of renewal theory" (It is a special type of the larger class of Volterra equations) Variants of this equation are encountered in the study of the growth of large populations or of the replacement of industrial equipment, where only expected values, not probabilities, are treated The *characteristic feature of a renewal equation is the appearance of the unknown* function in an integral expression of the form $\int_0^t m(t-u)f(u)du$

2 Solution of the renewal equation So far we have not made any special assumptions about the lifelength density $f(t)$ Generally speaking, we want to have available a variety of possible forms, capable of representing different possible types of lifelength, and simple to treat mathematically A family of density functions that meets both these criteria is

$$f(t) = \frac{c^{k+1}}{k!}t^k e^{-ct}, \tag{3}$$

where c is positive and k is positive (or 0, in which case $f(t) = ce^{-ct}$) If the lifelength T has a density of the form (3), then the mean and variance of T are respectively $ET = (l + 1)/c$ and $\sigma^2(T) = (l + 1)/c^2$. Conversely, if we know the mean and variance of T, we can attempt to represent the distribution of lifelengths by the density (3), taking $c = E(T)/\sigma^2(T)$ and (notice that l does not depend on the time unit) $l = [(ET)^2/\sigma^2(T)] - 1$.

The representation (3) seems reasonably like that observed in certain forms of bacteria where values of l in the neighborhood of 20 seem appropriate.[14]

If we use the form (3) and require that l be an *integer* (or 0) then it is possible to obtain an explicit solution of equation (2), although numerical evaluation of the solution is tedious. We shall not go into this but investigate the behavior of the solution of (2) when t is large without making special assumptions about $f(t)$.

3. Behavior of the first moment when t is large. It can be shown that *the expectation $m(t)$, satisfying equation (2), has approximately an exponential form when t is large. More precisely we can show that $m(t) \sim n_1e^{bt}$, that is*

$$m(t) = n_1e^{bt}[1 + d(t)] \tag{4}$$

where $d(t)$ is a function of t that goes to 0 as t increases and the constants b and n_1 are determined as follows.

(i) The integral $\int_0^\infty e^{-xt}f(t)dt$ is a function of the variable x. Since t is positive, this means that e^{-xt} gets smaller as x increases and hence so does the whole integral. When $x = 0$, the integral is $\int f(t)dt = 1$ and it can be shown that the integral goes to 0 when x increases. There is, therefore, a value of x which makes the integral equal to $\frac{1}{2}$ and this value is b:

$$\int_0^\infty e^{-bt}f(t)dt = \frac{1}{2} \tag{5}$$

(ii) The constant n_1 is given by

$$n_1 = \frac{1}{4b\int_0^\infty te^{-bt}f(t)dt} \tag{6}$$

We shall not go into the derivation of (4), (5), and (6), which can be obtained from works on the renewal equation, e.g., Feller.[7]

Example. Suppose that $f(t)$ has the form (3) with $l = 19$ and $c = 1$ when the time is measured in minutes. This is not far from the case with certain bacteria.[13, 14] The mean lifelength is thus $ET = 20/1 = 20$ minutes (see Section 2) and the standard deviation is $\sigma(t) = \sqrt{20} = 4.47$. Calculation shows that in this case

$$\begin{aligned} b &= 0.03527 \quad , \\ n_1 &= 0.7339 \quad , \end{aligned} \tag{7}$$

and thus for large t we have $m(t) \sim 0.7339e^{0.03527t}$. This exponential growth rate for the *expected values* reminds us of the Malthusian law of geometric increase for large populations.

4. Fluctuations. So far we have discussed only mean values, and it is important to consider how important are fluctuations around the mean. The first step in this direction is to evaluate the standard deviation of $N(t)$. Without going through the detailed argument, we state that the procedure is to differentiate equation (1) again with respect to s and then set $s = 1$. On the left hand side of this equation we shall obtain $G''(1,t)$, which equals $EN^2(t) - EN(t)$ [see Section I.2, equation (4)]. On the right side we obtain integrals involving $G''(1,t-u)$, $G'(1,t-u)$—already known—and other known terms, and we thus have an equation that is similar to (2) and can be treated by similar methods. We give only the result for large t.

The standard deviation $\sigma(N(t))$ has approximately an exponential form for large t. More precisely, we have

$$\sigma(N(t) \sim n_1 \left(\frac{4J-1}{1-2J}\right)^{1/2} e^{bt}, \tag{8}$$

where n_1 and b are the same constants that appear in (5) and (6), and J is given by

$$J = \int_0^\infty e^{-2bt} f(t)\,dt \tag{9}$$

It is important to notice that the standard deviation grows at the same rate as the mean.

IV. Behavior of $N(t)$ as Time Increases

What is the behavior of the total number of cells $N(t)$ as t increases? Intuition suggests that once the population attains a moderate size, it should then grow steadily in a geometric fashion until the cells become crowded enough to hamper one another, at which point the applicability of our model ceases. We shall now investigate this idea more closely.

Unless otherwise stated, statements made about the population size $N(t)$ will refer to the mathematical model, so that we shall be free to make categorical statements without qualification. Some remarks will also be made about the realism of the model.

1. *The logarithmic phase.* If we plot the natural logarithm $\ln N(t)$, we obtain an increasing curve that has irregular random fluctuations when t is small but eventually smooths out and becomes almost a straight line. If we plot $\ln N(t)$ from another experiment independent of the first, we find the same behavior, the eventual straight line having the *same slope* as the first, but a *different intercept*. Figure 1 shows this behavior schematically, although the curves have been drawn smoothly, without trying to show the jumps corresponding to individual births. The period of linear growth of $\ln N(t)$ is called the *logarithmic phase*.[11]

Since $\ln [EN(t)]$ is, from (III.4), approximately $\ln n_1 + bt$ when t is large, we should expect the slope of the curve for $\ln N(t)$, if it settles down to anything at all, to settle down to the constant b defined by (III.5). Thus, when t is large, we should have approximately

$$\ln N(t) = bt + \text{a random variable independent of time} \tag{1}$$

Equation (1) says that when t is large, we should have $N(t) = e^{bt}$ times (a random variable independent of time).

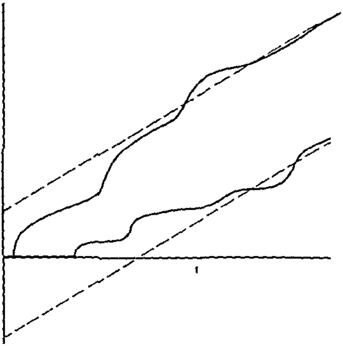

FIG 1 —Logarithm of the number of cells as a function of time

This suggests that we study the reduced random variable $N(t)/e^{bt}$ and it is also convenient to divide by the constant n_1 defined by (III 6) We then have the following result

Theorem 1 When t increases the random function $N(t)/(n_1e^{bt})$ approaches a limit N_0, and N_0 is a random variable that is, we have $N(t) \sim N_0n_1e^{bt}$

We cannot enter into the proof of Theorem 1 We mention, however, that the main step in the proof comes in showing that if t_1 and t_2 are large, then $N(t_1)$ and $N(t_2)$ are almost perfectly correlated

2 The distribution of N_0 The probability distribution of N_0 is now of obvious interest Since $EN(t)$ is approximately n_1e^{bt} when t is large, we see that $EN_0 = 1$ The next step is to relate the standard deviation of N_0 to the distribution of T *of a single cell If we go back to (III 8),* we see that when t is large the standard deviation of $N(t)/(n_1e^{bt})$ is $[(4J - 1)/(1 - 2J)]^{1/2}$ where J is defined by (III 9) Hence we have

$$\sigma(N_0) = \left(\frac{4J - 1}{1 - 2J}\right)^{1/2} \tag{2}$$

The following approximate rule proposed by D G Kendall[14] is also very useful *the standard deviation of N_0 is nearly equal to the coefficient of variation of the life length of one cell, $\sigma(T)/ET$* (We have scaled N_0 so that its mean is 1, if this had not been done the rule would still hold with $\sigma(N_0)$ replaced by $\sigma(N_0)/EN_0$) It is possible to deduce further facts about the distribution of N_0, but we cannot go into this here

Example Suppose we treat again the example of Section III 3 The quantity $\sigma(T)$, as given by (2), can be calculated to be 0.22 The coefficient of variation of T is $1/\sqrt{20} = 0.22$, in agreement with the above approximation

3. *A special case.* Let us consider the very special case where the lifelength density $f(t)$ has the form ce^{-ct}. This is the only case where all the distributions involved can be found explicitly, and is thus worth some attention, even though the assumed form is unrealistic. The treatment in this case is particularly simple because of the unique property of the exponential distribution that the death rate is independent of age (see Section II 1). This is the classical problem treated by Yule[22] in an application to the development of new species, and by Furry[9] in a problem in the physics of cosmic rays. In this case the generating function $G(s,t)$ can be shown to have the form

$$(3)\quad G(s,t) = \frac{s}{e^{ct} - (e^{ct} - 1)s} = e^{-ct}[s + (1 - e^{-ct})s^2 + (1 - e^{-ct})^2 s^3 + \cdots]$$

Since the probability $P[N(t) = k]$ is just the coefficient of s^k in the expression (3), we have

$$(4)\qquad P[N(t) = k] = e^{-ct}(1 - e^{-ct})^{k-1}$$

for $k = 1, 2, \cdots$. The expected value $m(t) = EN(t)$ is just e^{ct}. In this case, the density function for the limit $N_0 = \lim N(t)/e^{ct}$ can be determined exactly, it is just e^{-x}.

4. *Later stages of growth of the population.* If we are dealing with a population confined to a limited space, the logarithmic phase of growth cannot continue indefinitely, and new factors must be considered. By the time the cells begin to inhibit one another, it may be that there are enough present so that a mathematical model of the deterministic sort is appropriate, rather than treatment by probability theory. Thus one could perhaps treat the early stages of growth in the manner of preceding sections and treat the later stages by means of differential equations. This sort of procedure has been used by D. G. Kendall[1] in the mathematical treatment of epidemics. As far as I know, this possibility has not been explored for the growth of populations of cells.

Even in a population that is stationary because of crowding, it may be reasonable to follow the fortunes of an individual family (e.g., a family of mutants or a labeled family) in the manner of preceding sections. In this case we would modify the treatment so as to allow deaths as well as fissions.

V. THE AGE DISTRIBUTION FOR THE LOGARITHMIC PHASE

If we knew the age of each cell present at time t, we could construct the function $N(t,y)$, the number of cells whose age at t is y or less. The ratio $N(t,y)/N(t)$, considered as a function of y, is the proportion of the population aged y or less at time t; this proportion is itself a random variable. For obvious reasons, we call the function of y defined by $N(t,y)/N(t)$ the *age distribution*.

1. *The limiting age density.* When only a few cells are present, we should expect the age distribution to be quite irregular. On the other hand, after a long time period, when many cells are present, we might expect the age distribution to settle down to a definite limit, and this is the case.

As t increases, the age distribution $N(t,y)/N(t)$ converges, for each value of y, to the cumulative probability distribution function whose density is

$$a(y) = \frac{e^{-by}[1 - F(y)]}{\int_0^\infty e^{-by}[1 - F(y)]\, dy} = 2be^{-by}[1 - F(y)], \tag{1}$$

where b is the constant defined by (III.5) and $F(y)$ is the cumulative lifelength distribution for a single cell. We call the function $a(y)$ defined by (1) the *limiting age density*. Roughly speaking, we may say that when the population reaches a large size (but is still in the logarithmic phase), the fraction in the age range y_1 to y_2 is given by

$$\int_{y_1}^{y_2} a(y)\, dy \tag{2}$$

[Notice that $a(y)$ is a density, although the cumulative distribution $F(y)$ appears in the definition (1).]

To obtain the last expression in (1), we need to use the relation (III.5), and we also use the following relation, obtained from integration by parts:

$$\int_0^\infty e^{-bt}F(t)\, dt = \frac{1}{b}\int_0^\infty e^{-bt}f(t)\, dt = \frac{1}{2b}.$$

Let us examine the meaning of the formula (1). The positive constant b represents the population growth rate. Now if we deal not with a growing population but with a large stationary population, where each cell that disappears is replaced by just one new cell and not two, then it is known that the limiting age density is just $C[1 - F(y)]$, where C is a constant. The density in (1) contains the factor e^{-by}, which tends to increase the concentration in the smaller age range. This is because in an increasing population the age distribution is biased downward owing to the relatively large number of recently born objects, and the factor e^{-by} is the effect of this bias. The above facts about the age distribution can be deduced by methods similar to those employed in Chapters II and III.

2. *Illustration of the use of the age density: the fraction of cells in mitosis.* Before a cell completes dividing, it undergoes a period of change called *mitosis*. Hoffman, Metropolis, and Gardner[12] have considered the problem of determining the fraction of the population that is undergoing mitosis at a given time, using random sampling (Monte Carlo) methods. If the population has reached the point where the age distribution is described by the limiting age density $a(y)$, then we can use the results of the previous section to determine the fraction of the population in mitosis.

Suppose the cell lives a length of time T_1 before mitosis begins and spends a further time T_2 in mitosis. We might suppose the expected fraction of cells in mitosis at a given time to be $ET_2/(ET_1 + ET_2)$, but this overlooks the fact, discussed in Section 2, that multiplication of cells tends to give a bias towards younger cells and hence decreases the proportion of cells in mitosis below the value just given.

Suppose that T_1 and T_2 can be assumed to be independent of one another, with the respective densities $f_1(t)$ and $f_2(t)$. It is known from the theory of probability that $f_1(t)$ and $f_2(t)$ are related to $f(t)$ as follows:

$$f(t) = \int_0^t f_1(t-u) f_2(u)\, du$$

Let $F_1(t)$, $F_2(t)$, and $F(t)$ be the corresponding cumulative distributions.

Now consider a cell of age y. The probability that mitosis has begun, given that the cell has reached age y, is

$$\frac{F_1(y) - F(y)}{1 - F(y)} \tag{3}$$

Thus the expected fraction of cells in mitosis at a given time is obtained by multiplying the expression (3) by the age density $a(y)$ given in (1), and integrating over y.

We can evaluate the above integral in the following manner (for reasons of space we do not include a derivation here): *let R be the positive number determined by*

$$2 \int_0^\infty f_1(t) e^{-Rt}\, dt \int_0^\infty f_2(t) e^{-Rt}\, dt = 1 \tag{4}$$

Then the fraction of cells in mitosis after a long period of time is

$$2 \int_0^\infty e^{-Rt} f_1(t)\, dt - 1 \tag{5}$$

It should be pointed out, however, that the sampling experiments of Hoffman et al. indicate that it may take a long time for the fraction to settle down to this value.

Example. Suppose that $f_2(t)$ is the density for the time T_2 spent in mitosis, and that the time T_1 prior to mitosis is distributed as the sum of n independent random variables, each having the density $f_2(t)$. Then the fraction of cells in mitosis (the mitotic index) can be shown, using (5), to be $2^{1/(n+1)} - 1$. For example, if $n = 1$ (i.e., if T_1 and T_2 have the same distribution), then the mitotic index is $\sqrt{2} - 1 = 0.414$. If n is large, the expression $2^{1/(n+1)} - 1$ is almost equal to $\log_e 2/(n+1) = 0.693/(n+1)$, which is not much different from the expression equivalent to $\log_e 2/n$ given by Hoffman et al. (p. 177, footnote).[13]

VI. MORE COMPLICATED MATHEMATICAL MODELS

In order to treat types of cell multiplication different from binary fission, or to deal with assumptions less restrictive than the ones we have made hitherto, we can generalize our simple mathematical model in several directions. Some of these generalizations and their uses will now be indicated.

1. Case of two types. Let us suppose there are two types of cell, say 1 and 2, with lifelength densities $f_1(t)$ and $f_2(t)$. When a cell of type 1 splits, it has probabilities p_1, q_1, and r_1 of producing 2 cells of type 1, 1 of type 1 and 1 of type 2, or 2 of type 2, respectively, where $p_1 + q_1 + r_1 = 1$. Similarly, a cell of type 2 has probabilities p_2, q_2, and r_2.

To treat this situation, we use the double generating function

$$G_1(s_1, s_2, t) = \sum_{j=0}^{\infty} \sum_{k=0}^{\infty} P[N_1(t) = j, N_2(t) = k]\, s_1^j s_2^k$$

for the numbers $N_1(t)$ and $N_2(t)$ of types 1 and 2 at time t if initially there is one cell of type 1 There is a similar generating function G_2 for the case where there is initially one cell of type 2 We can deduce from reasoning similar to that which produced (II 3), the pair of equations

$$\begin{aligned} G_1(s_1\, s_2\, t) &= s_1[1 - F_1(t)] \\ &+ \int_0^t \{p_1G_1^2(s_1\, s_2\, t-u) + q_1G_1(s_1\, s_2\, t-u)G_2(s_1\, s_2\, t-u) \\ &\qquad + r_1G_2^2(s_1\, s_2\, t-u)\}f_1(u)\, du \end{aligned} \tag{1}$$

$$G_2(s_1\, s_2\, t) = s_2[1 - F_2(t)] + \int_0^t \{p_2G_1^2 + q_2G_1G_2 + r_2G_2^2\}f_2(u)\, du \tag{2}$$

Equations (1) and (2) can be treated similarly to the simpler equation of Chapter II although the possible modes of behavior are now more complicated than those of Chapter II

Example 1 Mutations Suppose type 1 is a normal form and type 2 is a mutant form If backward mutations are impossible we may represent this situation by the above model with

$$\begin{aligned} p_1 &= 1 - p & q_1 &= p & r_1 &= 0 \\ p_2 &= 0 & q_2 &= 0 & r_2 &= 1 \end{aligned}$$

Example 2 Correlations We can treat certain cases where there is correlation between lifelengths of mother and daughter cells or sister cells or both by appropriate choice of p s and q s in the above model For example suppose type 1 has a greater mean lifelength than type 2 Let us take

$$p_1 = p_2 = 1 - p \qquad q_1 = q_2 = 0 \qquad r_1 = r_2 = p$$

Then each birth will produce either two cells of the longer lived type or two of the shorter lived type with probabilities that are the same for either type of parent Here is a case with correlation between sisters but not between mother and daughter (Some data given by Powell[20] suggest that for certain bacteria this is the case, although the model just sketched may be too simple to deal with the real situation)

Cases where there are more than two types can be dealt with in a similar way, as can cases where fission is not binary but multiple

2 *More complicated kinds of reproduction* We have considered only a type of reproduction where fission is accompanied by complete disappearance of the old organism and the simultaneous appearance of new ones But a higher organism may produce progeny several times during its life as in the case of larger animals The mathematical treatment of this situation is somewhat more complicated than the case where all births occur at once and we shall not enter into it here beyond the statement that the mean and variance of $N(t)$ again satisfy equations of the renewal type

VII CONCLUDING REMARKS

The number of instances where probabilistic theories of population growth have been tested experimentally or used in the interpretation of experiments, as far as I know, is small As mentioned above, Kelly and Rahn[13] and Powell[20] have made direct observations of the lifelengths of bacteria The well known experiments of Luria and Delbruck[18] were interpreted by means of a probabilistic mathematical model to yield conclusions about bacterial mutations Further experiments are mentioned by Armitage [1] Some experiments on competing populations of beetles have been discussed in the light of probabilistic models by Neyman Park, and Scott [19]

It seems also true that some workers in biology feel that their thinking or argumentation is advanced by the use of mathematical models, even when direct experimental verification of the conclusions is difficult or impossible Thus, geneticists have used mathematical models to study the survival probabilities of mutants with a small advantage over the main population Such studies may at any rate be preferable to unquantitative speculation

The mathematical treatment given in this paper is based chiefly on work by Bellman and Harris[5] and Harris [10] Further mathematical treatment of population growth by probabilistic methods is given, in addition to the various references cited above, in papers by D G Kendall,[14-16] M S Bartlett,[3] and in the book of Bartlett [4] The recent book by Bailey[2] on the mathematical theory of epidemics should also be mentioned A mathematical treatment of regenerative processes has been given by Smith [21]

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Some Remarks on Changing Populations

By Heinz Von Foerster

Today we are beyond the stage where unaided observations will answer the questions we ask. The observer is surrounded by a battery of complex tools extending his senses far beyond their natural limits. Although these tools are not the objects of his observations, he must familiarize himself with their principles, techniques of operation, capacities and limitations, in order to make sure that what he observes is indeed what he wanted to observe. Since these tools nowadays are becoming more and more intricate and specialized, the manufacturers of these tools are kind enough to send along with their products an instruction manual which has to be carefully studied before the instruments are put to use. Of course, this instruction manual does not describe the forthcoming experiment—it describes the tool.

Although making observations and drawing inferences from these observations are two different arts, there is a similarity between the two which should not be overlooked.

We are also beyond the stage where simple reasoning permits us to draw inferences from the complexity of our observations. Today the interpreter of these data has access to a wealth of conceptual tools extending his power of reasoning far beyond his personal limits. Although these tools are not the objectives of his investigations, he has to familiarize himself with their principles, techniques of operation, capacities and limitations in order to make sure that what he investigates is indeed what he wanted to investigate. Since these conceptual tools nowadays are becoming more and more intricate and specialized, the manufacturers of these tools should be required to send along with their product an instruction manual which has to be carefully studied before the mathematics is put to use. Of course, the instruction manual does not describe the forthcoming interpretation of an observation—it describes the tool.

In the following remarks about populations and their behavior in various states of equilibrium I am attempting to describe a mathematical tool which is—so I sincerely believe—tailor made for the interpretation of some kind of observations that experimental hematologists are now making. In the instruction manual that comes along with this tool I will make all efforts to point out its underlying principles, technics of operation, capacities, limitations and the significance of the various built-in pointers, switches, trap-doors, mirrors, lenses and buttons.

POPULATIONS

Although everybody seems to have a good intuitive idea of what is meant by a population, one has to sharpen this concept considerably if one attempts to formulate statements about populations in mathematical language. The essential point which distinguishes a population from a mere set of elements is, in my opin

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ion, the particular property of the individual element of a population that it changes as time goes on. The most drastic changes an element can undergo are spontaneous appearance (birth) and spontaneous disappearance (death). Aside from these, there may be many other possible changes, such as location, appearance, activity, etc. that an individual element may make during its life. The description of a state in which elements may be found I shall call a state-description. Such a state-description may be very general, e.g. the red cells in the vascular system, or very specific, e.g. such and such a dividing cell in its n th generation. Since it is the business of all quantitative descriptions of populations to count the number of elements answering a particular state description, it is important to make this description as unambiguously as possible, because adding or subtracting a single property from this description may drastically change the number of elements under consideration. Although in many instances there may arise some doubt as to whether or not certain elements should be included in the population count, these cases may be dismissed if they constitute only a small fraction of the total population. If, however, the population is mainly made up of such fringe cases, it is advisable to postpone a numerical evaluation of this situation until we are in the possession of a calculus of ill-defined objects, which I fancy may be intriguing to develop. However, this discussion will be restricted to the simple case where indeed a sufficiently large number of elements can be found *which for most observers seem to answer a certain state-description.*

After such a state-description has been furnished, one may ask about the number of elements populating this state. Hence, two symbols are necessary to convey this situation, and I shall use N to present numbers of elements. If more than one state is to be discussed, I shall indicate this with a prefix $_sN$ where s is a number referring to the s th state-description in a catalogue of such descriptions. To give a simple example: in a 2-dividing, lossless population, where the state description is belonging to the n th generation, we have $_nN = 2^n {}_0N$. Should it be evident from the context which state one is talking about, the prefix may be dropped. In the following I shall discuss mainly some properties of populations defined by a single state-description, although the mathematical apparatus developed herein should allow me to use it for multi-state populations, as I will show later in some simple examples.

In order to represent adequately the properties of a given population, two more pieces of information with respect to a particular element are required. The one is a statement of how long this element has been in this state, which I shall refer to as the age of the element with respect to this state; the other one is the total time span an element may be observed in such a state, marked by the instant of its entry and the instant of its exit. This I shall call the duration of the element with respect to this state. Age and duration will be symbolized by the letters *a* and *D*, respectively.

Having introduced these temporal concepts, we may now ask about the number of elements ΔN, which can be found in a given age groups going from age a to age $a + \Delta a$, where the finite interval Δa may be chosen conveniently, say one year, one day, one minute, etc., depending upon the kind of elements under consideration. This number of elements per age interval Δa, will be called the finite age distribution, in symbols

$$\frac{\Delta N}{\Delta a} = \Delta N_a \tag{1}$$

After Δa has been chosen conveniently, a graphical representation of ΔN_a would result in a step function as indicated in figure 1a Clearly, the sum of the elements in all these age intervals should account for the total population

$$\sum_{a=0}^{a=a_{\max}} \Delta N_a = N \tag{2}$$

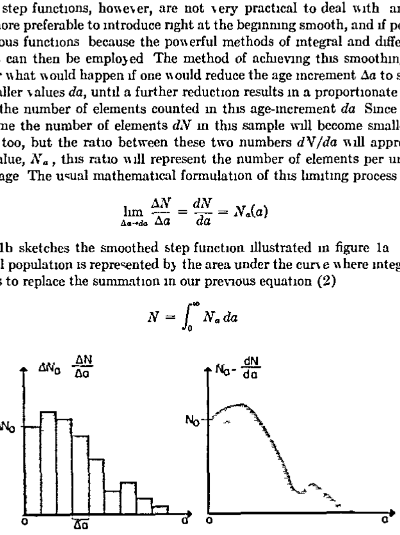
as indicated by the area under the step function If there is good reason that maximum age ($a = a_{\max}$) in this particular population is a doubtful concept, one may play it safely by letting $a_{\max}$ go to infinity

Such step functions, however, are not very practical to deal with and it is much more preferable to introduce right at the beginning smooth, and if possible continuous functions because the powerful methods of integral and differential calculus can then be employed The method of achieving this smoothing is to consider what would happen if one would reduce the age increment Δa to smaller and smaller values *da*, until a further reduction results in a proportionate reduction of the number of elements counted in this age-increment *da* Since at the same time the number of elements *dN* in this sample will become smaller and smaller too, but the ratio between these two numbers dN/da will approach a finite value, N_a, this ratio will represent the number of elements per unit age *at* that age The usual mathematical formulation of this limiting process is

$$\lim_{\Delta a \to da} \frac{\Delta N}{\Delta a} = \frac{dN}{da} = N_a(a) \tag{3}$$

Figure 1b sketches the smoothed step function illustrated in figure 1a Again, the total population is represented by the area under the curve where integration now has to replace the summation in our previous equation (2)

$$N = \int_0^{\infty} N_a\, da \tag{4}$$



(a) (b)

FIG 1 —Age distributions for (a) finite and (b) infinitesimal age intervals

Up to now I have tacitly assumed that by some means or another the age of an element could be directly determined Unfortunately, this cannot be done in most cases of those cell populations that we must deal with We have to go the other way around By this I mean that we would like to determine the duration of a particular state by counting the number of elements that can be found in this state at a given instant of time To briefly explain this, consider for the moment a state-description that would characterize, say, the erythrocytic series Since a particular element of this series undergoes changes in shape, our cell population N is made up of elements of different kinds which we may define as substates A, B, C, etc We are supposedly able to count the number of cells in these states

$$ {}_AN,\ {}_BN,\ {}_CN $$

such that

$$ {}_AN + {}_BN + {}_CN + \qquad {}_ZN = N \tag{5} $$

and from this, we would like to infer about the duration of the respective states

$$ \begin{aligned} D_A &= a_A - 0 \\ D_B &= a_B - a_A \\ D_C &= a_C = a_B \end{aligned} \tag{6} $$

where a_A, a_B, a_C is the age of termination of state A, B, C, respectively It sounds almost trivial to say that the answer to this question can readily be given, if the age distribution N_a is known Because then we have

$$ \begin{aligned} N &= {}_AN + {}_BN + {}_CN + \\ &= \int_0^{a_A} N_a\,da + \int_{a_A}^{a_B} N_a\,da + \int_{a_B}^{a_C} N_a\,da + \\ &= \int_0^{a_Z} N_a\,da \end{aligned} \tag{7} $$

Since N_a is unknown, we have just concluded a vicious circle, which usually is broken by making educated guesses as to the age distribution N_a One of the most popular assumptions of N_a is that it is a constant C (I call such populations equivivant and shall discuss this situation later in more detail) This assumption solves our problem immediately, because now

$$ {}_AN = \int_0^{a_A} N_a\,da = C\int_0^{a_A} da = C(a_A - 0) = CD_A $$

or

$$ \begin{aligned} D_A &= {}_AN/C \\ D_B &= {}_BN/C \\ D_C &= {}_CN/C \\ \cdots &\quad \cdots \end{aligned} $$

The only quantity missing is C, which may be found by determining the duration of at least one sub state or by other feasible methods

Another example of an assumed age distribution should be discussed, in order to make clear that whenever we choose a particular age distribution we have a certain mechanism in mind which may produce this distribution Imagine a 2 dividing population, where ${}_iN$ is the determinable number of elements in the i th generation What is the duration D_i of this sub state? The age-distribution is given at once by

$$N_a = N_0 2^{a/\tau} = N_0 e^{(a/\tau)\ln 2} = N_0 e^{\lambda a}$$

where $\tau = \ln 2/\lambda$ is usually called the doubling time of this population From (6) and (7) we have

$${}_iN = N_0 \int_{a_{i-1}}^{a_i} e^{\lambda a}\,da = \frac{N_0}{\lambda} e^{\lambda a_{i-1}} (e^{\lambda D_i} - 1)$$

whence

$$D_i = \frac{1}{\lambda} \ln\left[1 + \frac{{}_iN\lambda}{N_0} e^{-\lambda a_{i-1}}\right]$$

Since ${}_iN$ has been counted, a_{i-1} is given by

$$a_{i-1} = D_0 + D_1 + D_2 + \cdots + D_{i-1}$$
$$a_{-1} = 0$$

The only quantity missing is τ, or $\lambda = \ln 2/\tau$, which may be found by determining the duration of at least one sub state or by other feasible methods

I hope that these two examples helped to demonstrate the central position of age-distribution and their underlying mechanisms in the study of cell populations In the following I shall try to present methods of how one may get an insight into these mechanisms by making observations about numbers of elements in certain states or, if assumptions of these mechanisms are made what are the parameters that will describe completely the resulting populations

POPULATION SAMPLES

There are two main objectives underlying population studies One consists of deriving statements about a population as a whole from observations of the life history of its elements Demography or ecology are examples The other one consists of attempting to make conclusions about the elements by observing the behavior of the population as a whole We are mostly concerned with this side of the problem

The former approach is taken by the Bureau of Census deriving information about the behavior of the population as a whole by questioning each element of this population, whether or not it answers a certain set of state descriptions This is certainly a complete method with which to derive statements about the population under consideration In certain cases it may suffice to question only a "representative sample" of the population if one has made sure that the elements of this sample have been selected by a true random process, i e , no selective principle should have sneaked into the sampling method, and if this sample is sufficiently large in order to be considered a representative sample of the whole population The methods by which we may answer the question of how truly do the concise properties of this sample population reflect the properties of the population as a whole are the methods of statistics They have been developed to a high degree of perfection further elaboration of this topic must be left to the statistician The point I want to stress is that in this approach one has access to the individual element and by making records of its life history one may derive statements of the population of which this element is a member

In our situation however the individual element is in most cases not directly accessible for questioning In spite of this fact we would like to gain the most knowledge possible about its habits life history and whereabouts In this situation one hopes that by studying the population as a whole—or a representative sample of it—one may be able to derive statements about a typical element Obviously this approach is the inverse of the one discussed just before and the methods available to tackle this problem are to my knowledge by far less developed than in the previous case Unfortunately the problems we are dealing with—as I understand them—are just of that nature We would like to answer the question What does a typical leukocyte do? tacitly assuming that there is such a thing On the other hand we would reject the question What does a typical African do? as being ridiculous unless we permit noncommittal generalities as answers e g He lives in Africa where even those may be wrong

Keeping the problematic nature of our question in mind we see no other way to attempt to answer it but to study the population as a whole Since in almost all cases this too is impossible we have to restrict our aspirations usually to the study of relatively small samples hoping that these may serve as representative samples from which conclusions can be made about the properties of the population as a whole Having arrived at those we wish to derive statements about the behavior of typical elements of this population

There are in principle two fundamentally different methods by which samples of a population can be obtained The discussion of certain parameters that may suffice to describe these samples the populations they represent and their relationships with each other will make up the main core of this chapter

1 *Congenerate Sample*

Assume that we take a large number N_0 of newborns place them into an ideal environment and count in subsequent time intervals the surviving elements No other elements except those of the sample are admitted and no element is supposed to be removed from this environment Should this sample reproduce its offspring is assumed to be distinguishable from the parent population I hasten to explain the different terms I put under quotation marks The terms newborn and survival refer to a particular state If this state happens to be being alive then these words can be taken literally An element that undergoes drastic changes may be called a newborn with respect to a particular state whenever it assumes a shape that is defined by the appropriate state-description Similarly if an element loses the properties that make it an element of a particular state it does not necessarily mean that the element died We may call this transmutation or loss of identification By ideal environment I simply mean absence of toxic agents and sufficient space and food in order to avoid interaction of the elements

It is clear that this sample does not constitute a population in the usual sense, since at each instant there is only one particular age group available We can, however, construct with this sample a fictitious population by imagining that we would inject into this environment newborns at a constant rate of N_0 The age group distribution N_a of this population would turn out identical with the time plot of the original congenerate sample, only that the abscissa has become age instead of time Since N_0, the number of newborns, is arbitrary a much more significant function is obtained if all values N_a are divided by N_0 This function I shall call the ' intrinsic life function ' φ

$$N_a = N_0\varphi(a), \tag{8}$$

a somewhat arbitrary sketch of which is given in figure 2a Obviously, this func-

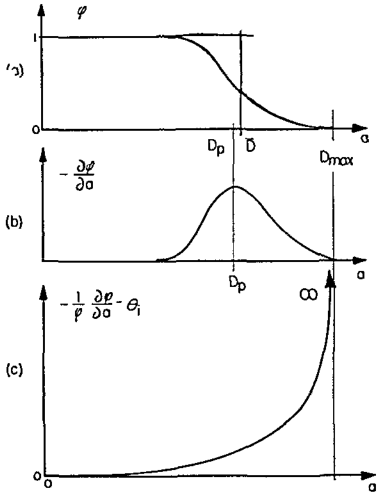


FIG 2 —Intrinsic life function (a), duration distribution (b), and intrinsic loss factor (c), for a congenerate sample population

tion must start for $a = 0$ with $\varphi = 1$, since $N_a(0) = N_0$ and $N_0/N_0 = 1$ Another important property of this function may be mentioned namely that nowhere can it display a slope that is directed upward The slope of this function must always be negative

$$\frac{\partial\varphi}{\partial a} \leq 0 \tag{9}$$

This property is derived from the fact that we did not permit newcomers in age groups different than $a = 0$ to join this population and that the rate at which we fed new elements into this population remained constant A close relative of this function enjoys wide popularity and carries many different names Life expectancy is one of them because for a particular age a the corresponding value of this function may be said to give the probability of reaching at least that age I did not want to give my function φ any one of those names because of the particular conditions under which φ has been obtained namely the assumption of the idealized environment The purpose of this assumption is to separate environmental causes of the termination of a certain state as much as possible from genetic causes Life expectancies usually refer to a given environment with all its particular hazards.

Let me briefly discuss some other properties of the intrinsic life function First of all it may be argued that this function may assume different shapes at different times In other words φ should be considered not only as a function of age alone but also of time

$$\varphi = \varphi(a\ t) \tag{10}$$

This is certainly true On the other hand my somewhat idealized setup in order to establish φ would permit me to assume that the temporal changes of φ would be small indeed during the sojourn of an element in the state under consideration Hence, if we measure time t in multiples of maximal durations D_{max}, of this state I propose that

$$\frac{\partial\varphi}{\partial(t/D_{max})} \ll 1$$

or

$$\frac{\partial\varphi}{\partial t} \ll \frac{1}{D_{max}} \tag{11}$$

Since it may suffice to observe not too many generations in order to establish φ the temporal variations of this function may be neglected in the following remarks, unless particularly referred to

Two other functions may be directly derived from our intrinsic life function One indicates the *absolute* loss of elements at a particular age and is given by

$$\delta(a) = -\frac{\partial\varphi}{\partial a} \tag{12}$$

Multiply δ with N_0 and you have the number of elements lost per unit time at a given age. Figure 2b sketches δ as derived from φ (fig. 1a). Since "the number of elements lost at a particular age, a," means exactly the same as saying "this is the number of elements that were maintained in this state until they reached the age a, at which they disappeared," the function δ presents also the percentage of elements having a certain duration D, if the abscissa is marked D instead of a. In other words, δ gives the duration distribution. The most probable duration D_p is found, of course, where this function has a maximum.

The other function we may derive from φ indicates the *relative* loss of elements at a particular age and is given by

$$\theta_i(a) = -\frac{1}{\varphi}\frac{\partial \varphi}{\partial a} \tag{13}$$

This function is sketched in figure 2c, and, since we will soon discover its important position in our population theory, I shall refer to it as the 'intrinsic loss factor.' θ_i represents the number of elements lost per unit time at age a, divided by the number of elements having survived at this age.* From this and (13) we see at once with (12) that

$$N_a\theta_i = -N_0\varphi\frac{1}{\varphi}\frac{\partial \varphi}{\partial a} = N_0\delta, \tag{14}$$

i.e., the number of elements lost per unit time at a given age. Clearly, the number of elements lost per unit time for the whole population is

$$\left(\frac{dN}{dt}\right)_{\text{loss}} = \int_0^\infty N_a\theta_i da \tag{15}$$

Inserting into (15) the expressions for θ_i and N_a from (13) and (8), respectively

$$\left(\frac{dN}{dt}\right)_{\text{loss}} = -N_0\int_0^\infty \varphi\frac{1}{\varphi}\frac{\partial \varphi}{\partial a}da = -N_0\int_1^0 d\varphi$$

$$= -N_0(0-1) = N_0$$

That means, that in our artificial population the total losses per unit time are equivalent to the 'newly born, N_0.' This should not surprise us, because this population was made up that way, by constantly feeding congenerate samples at the rate N_0 into our idealized environment, keeping the system in a steady state.

Before concluding the discussion on the congenerate sample, I would like to say a few words about averages. One we mentioned already, D_p, the most probable duration of this state. Another average, the "mean duration," $\bar{D}$, is usually found by calculating the area under the φ function and constructing a rectangle with same area and unit height (fig. 2a). The length of this rectangle represents the mean duration

$$\bar{D} = \int_0^\infty \varphi da \tag{16}$$

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[*] A close relative of this function is known as 'mortality.' There are however subtle differences between θ_i and mortality, which will not be discussed here.

Since the total population is given by

$$N = \int_0^\infty N_a da \tag{17}$$

we have with (8) and (16)

$$N = N_0 D \tag{18}$$

If a population's age-distribution can be closely approximated by a rectangle, I shall call this an "equivivant population" This means that

$$\varphi = 1 \quad 0 \leq a < D \tag{19a}$$

$$\varphi = 0 \quad D \leq a$$

and

$$D = D_p = D_{\max} = D, \tag{19b}$$

or all elements share exactly the same fate This assumption may, in some cases, indeed represent a good approximation to the real situation[2, 8]

Finally, I would like to give an expression for a "mean intrinsic loss coefficient"

$$\theta_i = \frac{\int_0^\infty N_a \theta \, da}{\int_0^\infty N_a da} = \frac{1}{D} \tag{20}$$

which can easily be verified by using Equations (15) and (16)

We have discussed the properties of this somewhat artificial population sample in greater detail than it may seem to deserve However, most of the ideas presented here will be applicable to the other sample to which we now shall turn our attention and which displays by far more realistic features than the one just discussed

B Contemporary Sample

Assume a population consisting of a large number $\mathfrak{N}$ of elements At a particular instant we collect from this population a sample consisting of N elements, such that this sample may be considered a proper representation of the original population Since all elements have been collected at the same instant, I call this a "contemporary sample" So far as I understand existing laboratory technics, this sampling method seems to fall into the realm of realizability Unfortunately, I am going to leave this realm already by proposing to examine the elements of this sample population with respect to their age, and to plot the number of elements ΔN_a counted in a certain age group $a \rightarrow a + \Delta a$ against the age a The stepfunction so obtained may be smoothed according to the procedure outlined before, and the resulting curve $N_a(a)$ shall be called the "apparent age distribution" Dividing these numbers N_a by N_0, the number of elements found at age zero, we arrive at a function ψ which is the counterpart of the intrinsic life function φ discussed in the previous section

$$N_a = N_0 \psi \tag{21}$$

This function ψ I call "apparent age distribution function," a somewhat arbitrary sketch of which is given in figure 3

Two things about ψ may be said at once First, there is no reason why this function should display only negative slopes, as is the case with the intrinsic life function [see Equation (9)] Since ψ reflects all environmental influences which may have acted upon our population in the past, its shape may be quite arbitrary

$$\frac{\partial \psi}{\partial a} \lesseqgtr 0 \tag{22}$$

Second, there is no good reason to assume that, if after a short time a second sample N' is taken from the original population $\mathfrak{N}$, this second sample should give exactly the same age distribution as the first one In other words, we can not neglect temporal changes of ψ as we could with φ

$$\frac{\partial \psi}{\partial t} \neq 0 \tag{23}$$

Hence

$$N_a(a,t) = N_0(t)\ \psi(a\,t) \tag{24}$$

It seems that with this expression we have arrived at a dead end in our speculations. Not only that we introduced the unrealistic inspection of elements with respect to their age, instead of inspecting them with respect to distinguishable substates Moreover, the whole situation seems to go out of hand due to the additional complication that was brought about by introducing time as another nasty variable By my pointing out this difficulty and by my proposal of how to resolve it, I have arrived at the core of my presentation

Instead of giving up at the point where we may observe temporal changes in

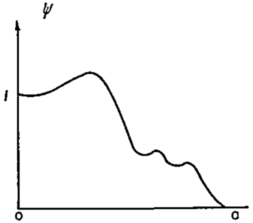
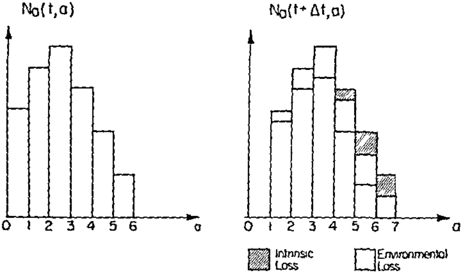


FIG 3 —Apparent age distribution function for a contemporary sample population



(a) (b)

FIG 4 —Temporal change of age distribution due to aging and environmental losses

our sample population, we may ask "What can we learn from such fluctuations?" In order to follow with greater ease the subsequent reasoning, figure 4 may be consulted Figure 4a is a sketch of an apparent age distribution as it may have been observed at a certain time t Sketching it as a stepfunction is irrelevant to the argument, and serves only to facilitate recognition Now suppose while we let a small time increment Δt pass by, nothing would happen to the elements, but the elements grow older by a small age increment Δa This is to say that the age distribution $N_a(t+\Delta t,a)$ at time $t+\Delta t$ (fig 4b) would be the same as it was at time t, if everybody were Δa younger

$$N_a(t+\Delta t,a) = N_a(t\ a-\Delta a) \tag{25}$$

Removing the idealization expressed in the above equation and returning to reality, we can recognize at once two factors that have acted upon our population while it was exposed to the world for a time increment Δt One factor is the *loss of elements in each age group, due to the intrinsic loss while aging an amount* Δa, as discussed before Hence, we should subtract from the right hand side of Equation (25) at least the intrinsic loss $N_a\theta_i\Delta a$ At the same time, however, we should account for another factor, namely, the losses that each age group may suffer from environmental interaction Introducing an "environmental loss coefficient" θ_e, in analogy to our intrinsic loss coefficient θ_i, Equation (25) becomes now

$$N_a(t+\Delta t,a) = N_a(t,a-\Delta a) - N_a\theta\Delta a, \tag{26}$$

where I have combined θ_i and θ_e to a compound loss factor θ

$$\theta = \theta_i + \theta_e \tag{27}$$

Expanding the two expressions of N_a around t and a, we obtain

$$N_a(t+\Delta t,a) = N_a(t,a) + \frac{\partial N_a}{\partial t}\Delta t + $$

$$N_a(t,a-\Delta a) = N_a(t,a) - \frac{\partial N_a}{\partial a}\Delta a + $$

Inserting these into (26), neglecting higher powers in Δa and Δt, and realizing that $\Delta a = \Delta t$, we arrive at the important conclusion

$$\frac{\partial N_a}{\partial a} + \frac{\partial N_a}{\partial t} = - N_a\theta \qquad (28)$$

In order to give a complete appraisal of this differential equation, much more should be said than I could possibly say in this article. I would like, however, to mention only a few points that may illustrate in how many ways this equation can be put to use to help us in analyzing our data. A brief explanation in words of the meaning of the above equation may help to get these points across.

Let us consider the following experimental situation. At subsequent time intervals t_0, t_1, t_2, t_3 contemporary sample populations N, N', N'', N''' are collected from the population $\mathfrak{N}$ under study. In each of these sample populations an age-group count N_a, N_a', N_a'', N_a''' is made. These data can be recorded in two different ways. First, one may plot the apparent age distribution observed at different times t_i, as discussed already in great detail. This would lead to a representation as sketched in figure 5a, which is denoted $N_a(t_i, a)$. Since along each curve the parameter "time" is constant, slopes on these curves represent $\partial N_a/\partial a$, the first term in Equation (28). The other way to represent the data collected in our experiment consists of plotting for each age group a_i the number of elements as a function of time. This would lead to a representation as sketched in figure 5b, which is denoted $N_{a_i}(t,a_i)$. Since along each of these curves the parameter "age" is constant, slopes on these curves represent $\partial N_a/\partial t$ the second term in Equation (28). Hence, the simple wisdom underlying the Differential Equation (28) is that the sum of the variations of elements in

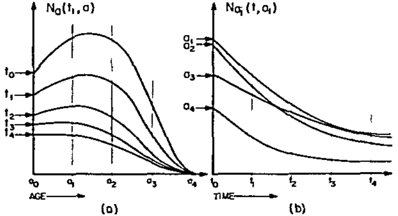


FIG 5.—Two ways of representing temporal variations of age distributions (a) for fixed time, number of elements are plotted against age, (b) for fixed age number of elements are plotted against time

each age group with respect to time as well as age is equivalent to the total gain in elements of this age group *

The first point that I could make of how to use Equation (28) to help us in the interpretation of our data would refer to the experiment just discussed the compound loss coefficient θ can be determined by graphical or numerical methods applied to our data Calculating the slopes in both representations (fig 5a, b) and adding them up we find $-\theta(a, t)$ Depending upon whether we have a pet philosophy concerning mechanisms determining either θ_i or θ_e, it is possible to determine either θ_e or θ_i by merely subtracting the assumed function from the observed θ

It also should be pointed out that Equation (28) establishes a connection between our intrinsic life function φ and the apparent age distribution N_a Using (27) and the definition of θ_i (13), Equation (28) can be rewritten

$$\frac{1}{N_a}\frac{\partial N_a}{\partial t} + \frac{1}{N_a}\frac{\partial N_a}{\partial a} + \theta_e = \frac{1}{\varphi}\frac{\partial \varphi}{\partial a} \tag{29}$$

Assume that our experiment would have shown that $\partial N_a/\partial t = 0$ This would have become apparent by the observation that the curves $N_a\,(t,a\,)$ (fig 5b) turned out to be just straight, horizontal lines Such a situation would indicate that our population $\mathfrak{N}$ is in a steady state Under these circumstances Equation (29) can easily be integrated, giving the connection between φ and ψ directly

$$\varphi = \psi \exp \int_0^a \theta_e da \tag{30}$$

Remember, ψ has been observed ($\psi = N_a/N_0$), hence φ could be established if the environmental removal mechanism of elements θ_e is understood, or vice versa The most important message of Equation (30) is that if, and only if, the environmental loss coefficient vanishes for all age groups ($\theta_e = 0$),† intrinsic life function φ and apparent age distribution function ψ become equivalent

$$\varphi = \psi \tag{31}$$

This is to say that if φ is assumed to be equivivant (Equation 19), ψ will be equivivant only, if no fatal" environmental influences on individual elements are exerted as long as they are in the state under consideration Should environmental influences occur, and equivivancy of φ is postulated, we have with (30) and (19)

$$\begin{aligned} &\psi = \exp\left(-\int_0^a \theta_e da\right), \qquad \varphi = 1, \qquad 0 \leq a < D \\ &\psi = 1, \qquad \varphi = 0, \qquad D < a \end{aligned} \tag{32}$$

Putting it differently If equivivancy of ψ is postulated, three basic assumptions with respect to the population as a whole have been made They are (1) steady state, (2) φ is equivivant, (3) no environmental losses $\theta_e = 0$

On the other hand, if a particular removal mechanism seems to have attractive

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* Gain is negative loss of course

† θ_e is assumed to be a continuous function

plausibility, ψ can be obtained at once, if a steady state should prevail and we have some idea about φ Let us take as an example a removal mechanism that would act randomly on all elements, irrespective of their age, number, or other properties This implies that θ_e is a constant

$$\theta_e = \lambda \tag{33}$$

Furthermore, let us assume, for simplicity, that φ is equivivant Since with θ_e given the integration in the exponent of Equation (32) can be carried out we have

$$\begin{aligned} \psi &= e^{\lambda a}, & \varphi &= 1, & 0 &\leq a < D \\ \psi &= 0, & \varphi &= 0, & D &\leq a \end{aligned} \tag{34}$$

If in this example λ is sufficiently large (strong environmental interaction) such that

$$e^{\lambda D} \ll 1 \tag{35}$$

there would be a very small chance indeed to ever detect the cutoff of the tail of the e function at $a = D$ Hence, the intrinsic duration D of elements in such an environment would escape notice The calculated mean duration of elements in this environment is in accordance with (16), given by

$$D = \int_0^D \psi da = \frac{1}{\lambda}(1 - e^{-\lambda D}) \tag{36}$$

hence, with approximation (35)

$$D \approx \frac{1}{\lambda} \tag{37}$$

That is, the calculated mean duration turns out to be the time constant of the removal mechanism, and we are left in the dark with respect to the intrinsic duration of the elements outside of this hazardous environment Whether or not this example may serve as an illumination on the controversial attempts to determine ‘ life span” of white blood cells I must leave to physiologists Doubtless once in the bloodstream, the white cell does not enjoy an easy life It is conceivable that it rarely sees its end due to mere aging after a natural life span D The life spans" we usually measure may be the time constants of the removal mechanism which in turn could depend on many external factors On the other hand the red blood cell, with its smooth surface and its being small enough to easily pass through the capillary bed, will be little affected by environmental hazards Making the same assumptions as before with respect to steady state and intrinsic life function, but with the one difference that λ should be sufficiently small (weak environmental interaction), such that

$$e^{-\lambda D} \approx 1 - \lambda D, \tag{38}$$

we obtain at once from (36) the mean life span of such a cell

$$\bar{D} = \frac{1}{\lambda}(1 - (1 - \lambda D))$$

hence

$$D = D \tag{39}$$

That is the calculated mean duration turns out to be the intrinsic life span of this cell and the environmental removal mechanism has no chance of showing up

Again I leave to physiologists to decide whether or not this example may serve as an illumination on the successful attempts to determine the life span of red blood cells What I want to stress here in these somewhat over-simplified examples is the point that however we want to turn our problem certain assumptions about the mechanisms involved have to be made in order to make up for our ignorance of parameters which are necessary to make complete statements about the populations under consideration Since the physical aspects of these mechanisms can be studied in some instances guessed and in most cases tested I propose to discuss these processes whose comprehension would resolve many a mystery I shall try to clarify this at the end of my paper In order to make this chapter on population samples complete I shall have two points to make

The first of these points leads us back to our fundamental partial differential equation (28) I want to mention briefly that such differential equations generally can be solved Without going into the mathematical detail I present you at once with its solution

$$V_a = \varphi \exp\left(-\int_0^a \theta_e da\right) \sum C e^{\lambda \quad a} \tag{40}$$

At first glance this may look frightening but closer inspection reveals that the factor in front of the summation sign is already known to us It is Equation (30), only that ψ is expressed through φ instead of the other way around Since Equation (30) was found under steady state condition the infinite sum must represent the transients that the population will undergo if steady state conditions are perturbed or permanently removed Indeed one sees time appearing in the exponent which indicates that the effects of these perturbations will die or grow exponentially with time depending on the other parameters λ and C_i in this expression Their values will depend on the initial conditions and on the way in which new elements are introduced into the population This brings me to the second point I had to make in order to conclude this chapter namely the production process

Up to now we only mentioned the losses a population may suffer but we were not concerned with the question of how this population may sustain itself There are three ways by which elements could be fed into a population

1 By feeding newborns from an outside source (precurser) into this population at a rate of Γ elements per unit time

2 By feeding elements from an outside source of a particular age group into the appropriate age-groups of this population (release after sequestration) This however, can be managed by assigning to our environmental loss coefficient θ_e a negative value

3 By generation of offspring

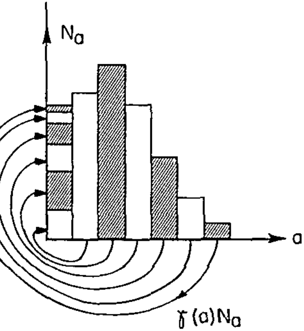

FIG 6 —The generation coefficient $\gamma(a)$

If we define a "generation coefficient" γ as the number of elements an element may produce while in a particular age group the number of elements produced by all elements of the population per unit time is, under steady state conditions* (fig 6)

$$N_0 = \int_0^\infty N_a \gamma da. \tag{41}$$

Since the production capacity of elements of different age may vary, the generation coefficient is assumed to be a function of age $\gamma(a)$ Although, on a more sophisticated level, one should consider γ to be a function of time too, $\gamma(a,t)$, we may pull the same argument we made previously for the intrinsic life function, and neglect temporal variations of this quantity

Using $N_a = N_0\psi$ and applying (30), the steady state result for ψ, Equation (41) becomes

$$\int_0^\infty \gamma\varphi \exp\left(-\int_0^a \theta_e da\right) da = 1 \tag{42}$$

Since this equation is obtained by assuming steady state, it may as well be interpreted as a condition which the three population-determining factors (1) intrinsic life function φ, (2) environmental loss coefficient θ_e, and (3) generation coefficient γ, have to fulfill in order to secure steady state † The importance of Equation (42) may be seen in the fact that for a population of elements characterized by their intrinsic life function φ, the *environmental* loss coefficient θ_e and the generation coefficient γ—essentially an *intrinsic* property*—have to be properly tuned together according to Equation (42) to guarantee stability of the system

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* For nonsteady state conditions see Appendix A

† Steady state conditions for particular cases are given in Appendix B

A simple example may illustrate this Let us consider an intrinsically equivivant population with random external loss and a production capacity independent of age

$$(a < D)\ \varphi = 1 \qquad \gamma(a) = \bar{\gamma}$$
$$(a \geq D)\ \varphi = 0 \qquad \theta_e(a) = \lambda$$

From Equation (30) we find at once the apparent age distribution function

$$\psi = e^{-\lambda a},$$

and Equation (42) tells us what value γ has to assume in order to guarantee steady state

$$\gamma \int_0^D e^{-\lambda a}\, da = 1$$

or, after integration

$$\gamma(1 - e^{-\lambda D}) = 1$$

If we speculate again about the influences of weak or strong removal mechanisms on the choice of $\bar{\gamma}$, postulating steady state, we find, using the previous approximations

$$\text{weak} \qquad (\lambda D \ll 1) \qquad \gamma = 1/D)$$
$$\text{strong} \qquad (e^{-\lambda D} \ll 1) \qquad \bar{\gamma} = \lambda$$

That is, if this population is exposed to a strongly acting environmental removal mechanism, the production rate has to adjust itself to the environmental demands If, however, the environmental influences are weak, it is only necessary that the product of the two intrinsic properties $D\bar{\gamma}$ is unity

The upshot of this section on population samples presents us with a dilemma, brought about by our attempt to derive the duration of substates by counting the number of elements in these states, the duration of which could be found if the age distribution were known—which, in turn requires the knowledge of the duration of the substates One way out of this dilemma would be to design experiments in which state durations could be measured directly without reference to the number of elements counted in these states This seems to lead to almost insurmountable difficulties Another way out is to hypothesize about the apparent age distribution function ψ As we know already, any hypothesis regarding ψ carries implicitely three other hypotheses along First, we have to assume steady state, second, a hypothesis about the intrinsic life function has to be made, third, we have to suggest a particular removal mechanism Since under normal conditions steady state seems to be a generally accepted feature of our cell populations I propose to study the effect on these populations caused by different assumptions about the removal mechanism postulating as a first approximation intrinsically equivalent elements As I shall how different removal mechanisms are reflected in the way populations return to their steady state after they have been temporarily perturbed These ways can be observed by counting elements in certain substates as a function of time The evaluation of these functions will produce additional information not obtained by the previously discussed methods of observation

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* A more general treatment would make the generation coefficient a function of environmental conditions Since these could change with time we should have $\gamma(a, t)$ Furthermore it may be noted that in sexually reproducing populations γ will depend on the availability of mates hence $\gamma(a, t, N_s)$

EQUILIBRIA AND PERTURBATIONS

Usually one distinguishes* three kinds of equilibria stability neutrality and instability

1 Stability is observed if a system possesses at least one preferred state S^* to which it will return after a temporary perturbation has been removed

2 Neutrality is observed if a system will eventually reside in any one of its accessible states S after a temporary perturbation has been removed

3 Instability is observed if a system will never reside in any one of its accessible states S after a temporary perturbation has been removed

The first two equilibria are usually referred to as steady states making proper reference to the temporal invariability of these states sacrificing however the important distinction between stability and neutrality It is just this point which will allow us later to distinguish between different kinds of removal mechanisms

Presently I would like to give a short description of the various methods in which our populations can be perturbed I shall distinguish again three different kinds parametric selective and random perturbations

1 *Parametric Perturbation*

In Equation (42) we have presented an important criterion for a population to be in a steady state Since this equation connects the three parameters γ θ_e and $\theta = -\partial\varphi/\varphi\partial a$ in a particular way these parameters have to assume certain values in order to satisfy the steady state condition Suppose a population is in a steady state but suddenly one of the parameters γ θ θ_e is changed Clearly the steady tate condition is now violated such a violation I shall call a parametric perturbation If a population is permanently perturbed in this way it may become unstable *unless* at least one of the three parameters is non linear, that is it is dependent upon the state of the population and thus will vary along with the variations of the population until—under certain conditions—it will assume a value that again satisfies Equation (42) and a steady state is secured again

2 *Selective Perturbation*

With this term I would like to describe an interference with the steady state by which in a specified way elements are added to or removed from individual age groups One way of visualizing this is by assuming an artificial removal mechanism θ_e^* at work, producing for a moment an artificial age distribution function ψ^* Since none of the parameters θ_e, θ_i, γ has been changed, the population will eventually resume a steady state The important question, "What is this state?" can only be answered if the three parameters are known On the other hand, if we are able to observe the new equilibrium after a given perturbation, statements about the parameters can be derived

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* For finer points of distinction see references 1 and

3 *Random Perturbation*

This is a special case of the perturbation just discussed Since no preference with respect to addition or removal of elements to or from certain age groups is now assumed, one may imagine an artificial removal mechanism temporarily at work, which is the same for all ages, hence, θ_e^* is a constant, independent of age, and the resulting artificial age distribution function ψ^* is proportional to the one obtained at steady state *Since none of the parameters* θ_i, θ_e, γ, *has been changed,* what has been said before with respect to the future of this population is applicable here too

TRANSIENTS

I hope I was able to make clear that in order to make a complete description of the fate of a population in a certain state, three mechanisms have to be understood. generation, intrinsic life function, and environmental removal mechanism In the difficult task of determining these three functions separately we are aided by the observation that if a steady state should prevail, these functions are not arbitrary, because they have to pass two check points One is given by Equation (30)

$$\psi = \varphi \exp\left(-\int_0^a \theta_e \, da\right), \tag{30}$$

which links the observed distribution function with the intrinsic distribution function, if the environmental removal mechanism is known The other one is given by Equation (42)

$$\int_0^\infty \gamma\psi \, da = 1 \tag{42}$$

which puts certain demands on the generation coefficient γ This leaves us still an ocean of functions to choose from, unless we add further restrictions on some of our functions in order to study the effects on our populations caused by particular choices of those mechanisms about which we are still free to speculate If the insight we have gained by applying this stratagem turns out to be relatively unaffected by the choice of our restrictions, this method of attack may have some merit

One of the restrictions I am tentatively introducing is intrinsic equivivancy This puts $\varphi = 1$ for all ages $a < D$, and it was already pointed out that this may not be too wild an assumption We are now free to speculate about γ and θ_e, where—among the two—our ideas about the production mechanisms seem to be less obscured than those about the removal processes There are in our case essentially only two ways of production to be distinguished proliferation and 2-division Although combinations of these two types may take place where cell populations characterized by particular states are spatially in close contact (jumping of intermediate divisions, divisions during differentiation, etc), those cases I shall tentatively dismiss in order to get the essential points across Having postulated mechanisms of generation, we are now free to speculate about removal processes separately By speculating' I mean that we may ask about which of the many environmental factors may be responsible for a premature termination of a particular state in which our elements reside We may cite pressure, adhesive forces, temperature turbulence, chemical agents phagocytosis, etc Whatever these agents may be, I propose to separate them into two major classes One, where the environmental agents will act on our elements in an accumulating fashion

$$\text{A)} \qquad \theta_e = F(a) \tag{43}$$

that is, the environmental hazards' will be a function of the time the element is exposed to these hazards, the other one, where in addition to environmental factors the very presence of the elements themselves influences their sojourn in the particular state under consideration

$$\text{B)} \qquad \theta_e = G(a, N_a) \tag{44}$$

Unfortunately, the involved mathematics does not permit quick proof of what I am going to say namely, that by making this class distinction we have at the same time divided the ensuing populations into those that will display the characteristics of a neutral equilibrium (A), and those that will show the features of stability (B)

In order to demonstrate these properties in two somewhat artificial examples I may refer to figures 7a and 7b Plotted here is the time-course of the number of elements in the zero-age group for the two different kinds of populations which have been subjected to exactly the same selective perturbations

In figure 7a, a population of type (A) with characteristic parameters

$$(a < D) \qquad \varphi = 1 \qquad \gamma - \gamma \qquad \theta_e = F(a) = 0$$

is initially composed of a linear age-distribution, in one case with many oldsters and few youngsters (solid line), in the other case with many youngsters and few oldsters (broken line) When the artificial restrictions that keep the population in this state are removed, the population will approach different steady states depending on its previous history

In figure 7b a population of type (B) is initially selectively perturbed exactly in the same ways as in our previous example The characteristic properties of this 2-dividing population are

$$(a < D) \qquad \varphi = 1, \qquad \gamma = 2\theta_e, \qquad \theta_e = \psi/D,$$

and we see that, as time goes on, the two different initial perturbations quickly disappear, and the population approaches a particular steady state which is independent of the previous history of the population

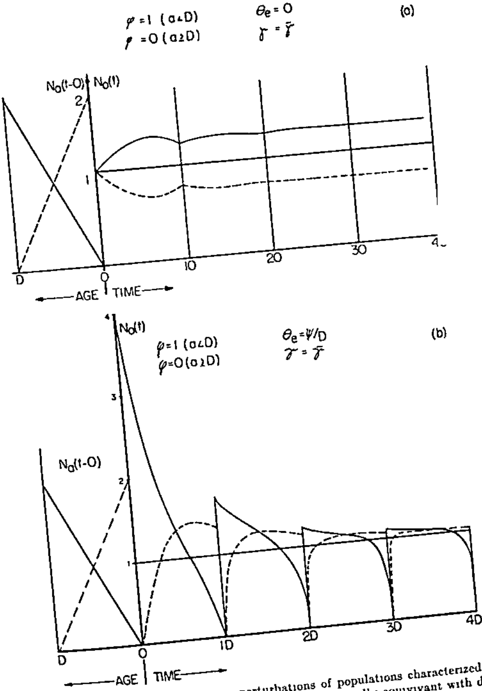

FIG 7.—Transients after selective perturbations of populations characterized by (a) neutral, (b) stable equilibrium The elements are intrinsically equivivant with duration D, and are 2 dividing after time D, feeding their offspring back into the population

In table 1, I have collected data about two families of special functions of $F(a)$ and $G(a,N_a)$ where, with C_n and K_n being constants:

$$F(a) = C_n a^n$$

and

TABLE 1

Assumption on φ γ	Intrinsically Equivalent 2-Dividing Elements	
	$\varphi = 1 \ (a < D)$ $\varphi = 0 \ (a > D)$	$\gamma = 2\theta$
Environmental Loss Coefficient θ_e	$\theta_e = C_n a^n$	$\theta_e = K_n a^n N_a$
Kind of Equilibrium	neutral	stable
Equilibrium condition	$C_n = \frac{(n+1)\ln 2}{D^{n+1}}$	$K_n = \frac{n+1}{N_0 D^{n+1}}$
Apparent Age Distribution Function ψ	$\psi = 2^{-(a/D)^n}$	$\psi = \frac{1}{1 + (a/D)^{n+1}}$
Total Number of Elements N	$N = A_n N_0 D$	$N = B_n N_0 D$
The Coefficients A_n and B_n	$A_n = \int_0^1 2^{-x^{n+1}}\, dx$	$B_n = \int_0^1 \frac{dx}{1 + x^n}$
Removal Production N_p	$N_p = \frac{N}{2\, D A_n}$	$N_p = \frac{n+1}{2K_n\, D^{n+1}}$

$$G(a, N_a) = K_n a^n N_a$$

Listed is their resulting apparent age-distribution function and the necessary equilibrium condition if 2-dividing elements are taken as a basis. The last line called "Removal Production" gives the rate of removal of elements from the particular state, which is characterized by properties just discussed. Since removal from this state could mean proliferation into a following state this double name seems to be justified.

The freedom of choice of the exponent n offers a wide variety of particular age distribution functions within these two families of populations. To mention only two quite popular cases, a neutral population will follow an exponential decay distribution if $n = 0$, but Gaussian distribution will result if $n = 1$

$$\left.\begin{array}{lll} n = 0 & \psi = e^{-\lambda x}, & \lambda = \dfrac{\ln 2}{D} \\[2ex] n = 1 & \psi = e^{-(x/\sigma)^2}, & \sigma = \dfrac{D}{\ln 2} \end{array}\right\} x = a$$

Finally, I would like to draw your attention to the possibility of rapid changes in the "removal production" of a stable population if parametric perturbations should occur. We have from table 1

$$N_p = \frac{n+1}{2K_n D^{n+1}} \tag{45}$$

The most obvious parameter that may change is D, the duration of the state, and if this is the last generation before maturation and differentiation, D is the last generation-time[7] The relative change of the production of this state with respect to a change in generation-time is

$$\frac{1}{N_p}\frac{dN_p}{dD} = -\frac{n+1}{D}, \tag{46}$$

which might be quite formidable, since D is relatively short Such a parametric perturbation could be produced by some D regulating agent

SUMMARY

I hope that these few examples have served as a stimulus for further exploitation of the theoretical outline I have tried to sketch On the other hand, I hope that these examples have not distracted from the central theme of my argument, namely, that in order to furnish a complete description of a population defined as elements in a certain state, it is necessary to specify three parameters that are all functions of the age of these elements with respect to the state under consideration. These functions cannot be obtained by counting elements while the population is in a steady state However, by introduction of perturbations the true nature of some of the parameters may be revealed Once in possession of the knowledge, at least of the functional structure of these parameters, not only an absolute time-scale for all substates of particular states can be estimated, but also an insight into possible physical mechanisms has been gained, which would permit the construction of physiologic models

APPENDIX A

Populations in Transient

If a steady state does not prevail, the number of elements in the zero age-group at time t has been produced by the population one instant dt before this time

$$N_0(t) = \int_0^\infty \gamma\, N_a(t-dt, a)\, da. \tag{A1}$$

Similarly, the population at time t will produce a zero age group observable an instant dt later

$$N_0(t+dt) = \int_0^\infty \gamma\, N_a(t, a)\, dt. \tag{A2}$$

Expanding $N_0(t+dt)$ and $N_a(t-dt,a)$ around t and subtracting $(A1)$ from $(A2)$, we obtain

$$\frac{dN_0}{dt} = \int_0^\infty \gamma \frac{\partial N_a}{\partial_t}\, da \tag{A3}$$

Utilizing (28)

$$-\frac{1}{N_0}\frac{dN_0}{dt} = \int_0^\infty \gamma \left(\frac{\partial \psi}{\partial a} + \psi\theta\right) da \tag{A4}$$

The relative rate of change of N_0 at a given time t has been reduced to properties of the population at that time With graphical or numerical methods the time course of N_0 can be determined step by step Today, of course, such problems can quickly be solved with the aid of high speed analog or digital computers For further information on populations not in steady state see also references 4 and 5

APPENDIX B

Steady State Conditions for Particular Cases

1 General

$$\frac{dN}{dt} = \left(\frac{dN}{dt}\right)_{\text{gain}} - \left(\frac{dN}{dt}\right)_{\text{loss}} = 0 \tag{B1}$$

$$\left(\frac{dN}{dt}\right)_{\text{gain}} = N_0 \int_0^\infty \gamma\psi\, da$$

$$\left(\frac{dN}{dt}\right)_{\text{loss}} = N_0 \int_0^\infty \theta\psi\, da, \tag{B2}$$

hence, with (41)

$$\int_0^\infty \gamma\psi\, da = \int_0^\infty e_d\psi\, da = 1 \tag{B3}$$

2 2-dividing population $\gamma = 2\theta_d$

From (B3) with $\theta = \theta_d + \theta_e$ we have

$$2\int_0^\infty \theta_d\psi\, da = \int_0^\infty \theta_d\psi\, da + \int_0^\infty \theta_e\psi\, da$$

hence

$$\int_0^\infty \theta_d\psi\, da = \int_0^\infty \theta_e\psi\, da \tag{B4}$$

3 Equivivant population

$$\left(\frac{dN}{dt}\right)_{\text{gain}} = N_0$$

$$\left(\frac{dN}{dt}\right)_{\text{loss}} = N_0\int_0^D \theta_e\psi\, da + N_0\psi(D) \tag{B5}$$

Steady state.

$$\int_0^D \theta_e\psi\, da = 1 - \psi(D) \tag{B6}$$

thus

$$\theta_s = -\frac{1}{\psi}\frac{\partial\psi}{\partial a} \tag{B7}$$

4 Equivalent 2 dividing population

$$\left(\frac{dN}{dt}\right)_{\text{gain}} = 2\psi(D) \quad N_s = N_0$$

$$\left(\frac{dN}{dt}\right)_{\text{loss}} = N_0 \int_0^D \theta_s\psi\, da + N_0\psi(D) \tag{B8}$$

Steady state

$$\int_0^D \theta_s\psi\, da + \psi(D) = 2\psi(D)$$

with (B6) or (B8)

$$\psi(D) = \frac{1}{2}$$

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Dynamics of Cell Proliferation and Isotope Incorporation into Deoxyribonucleic Acid

By Demetrios A. Rigas

THE RATE of isotope incorporation into deoxyribonucleic acid (DNA) has been used frequently in recent years in attempts to derive estimates of the life span of cells. However, the mathematical methods that have been used for this purpose are only approximate and are based on the assumption that a steady state is maintained.[1,5,6] A more detailed analysis of the kinetics of exponential proliferation in which no cell death occurs, and of the kinetics of the steady state, together with the use of the equations derived for the determination of the life span and of the generation time of cells, have been described.[8]

The analysis that is presented in this paper is a generalized theoretical treatment of the cell proliferation problem, which takes into account the effects of variables that may cause *growth*, *regression*, or maintenance of a *steady state* in terms of the number of cells.

I. Dynamics of Cell Proliferation

In the theoretical analysis of this problem, one can bypass important physiologic and biochemical questions, such as whether or not cells have the innate ability either to divide or to differentiate and die. The important fact is that some cells may die while others divide again. The probabilistic model whereby a group of cells formed at one time has a probability p of dividing again and a probability $(1 - p)$ of dying is adequate for our purpose. The equations derived on the basis of this model are applicable, regardless of the nature of the factors (innate or not, intracellular or extracellular) that bring about cell division, differentiation, or death. In this analysis, we shall refer to those cells that divide again as *cells destined to divide*, and to those that die as *cells destined to die*.

In the generalized nonsteady state of cell proliferation, it should be taken into consideration that the characteristic constants that appear in the analysis of the steady state[8] vary with time. The following functions thus enter this analysis:

$p_{(t)}$ = the probability that cells formed at time t will ultimately divide again

$I_{(t)}$ = the mean generation time of cells formed at time t

$g_{(t)}$ = the mean number of cells formed per mitosis at time t

In addition, for the cells *destined to divide* which are formed at time θ, the probability that they will not divide prior to a later time t, will be considered to vary not only with the age of the cells, $t - \theta$, but also with the time of their formation, θ, and will be represented by $\pi_{(t,\theta)}$. Similarly for the cells destined to die

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which are formed at time θ, the probability of survival up to time t will be represented by the function $\phi_{(t-\theta,\theta)}$

A Generalized Nonsteady State

1 *Cells destined to divide* The rate of formation of the *cells destined to divide* is proportional to the number of these cells, $P_{(t)}$, which are present This proportionality, however, may change with time, thus being a function $\sigma_{(t)}$, i e ,

$$\frac{dP_{(t)}}{dt} = \sigma_{(t)}P_{(t)} \qquad \text{or} \qquad \frac{dP_{(t)}}{P_{(t)}} = \sigma_{(t)}\,dt \tag{1}$$

Integrating from zero to t, we obtain

$$P_{(t)} = P_0 \exp \int_0^t \sigma_{(t)}\,dt, \tag{2}$$

where P_0 is the number of such cells present at zero time To express $\sigma_{(t)}$ in terms of $p_{(t)}$, $I_{(t)}$, and $g_{(t)}$, we resort to the following reasoning Suppose that the values of these functions at a time t remain constant from then on Under these conditions the number of *cells destined to divide*, $P_{[t+I(t)]}$ that will be present at time $t + I_{(t)}$ is

$$P_{[t+I(t)]} = P_{(t)}p_{(t)}g_{(t)} = P_{(t)}\,e^{\sigma(t)I(t)}$$
$$\therefore\ \sigma_{(t)} = \frac{\ln\,[p_{(t)}g_{(t)}]}{I_{(t)}} \tag{3}$$

Substituting in equation (2) we obtain

$$P_{(t)} = P_0 \exp \int_0^t \frac{\ln\,[p_{(t)}g_{(t)}]}{I_{(t)}}\,dt \tag{4}$$

2 *Cells destined to die* The rate of formation of these cells, $dM_{(t)}/dt$, also will be proportional to $P_{(t)}$ and can be expressed in a similar way

$$\frac{dM_{(t)}}{dt} = \lambda_{(t)}P_{(t)} = \lambda_{(t)}P_0 \exp \int_0^t \frac{\ln\,[p_{(t)}g_{(t)}]}{I_{(t)}}\,dt, \tag{5}$$

and after integration

$$M_{(t)} = M_0 + P_0 \int_0^t \lambda_{(t)} \left\{\exp \int_0^t \frac{\ln\,[p_{(t)}\,g_{(t)}]}{I_{(t)}}\,dt\right\} dt, \tag{6}$$

where M_0 is the number of such cells present at zero time To express $\lambda_{(t)}$ in terms of $p_{(t)}$, $g_{(t)}$, and $I_{(t)}$, we suppose that the values of these functions at a time t remain constant from then on Thus we have

$$M_{[t+I(t)]} = M_{(t)} + P_{(t)}g_{(t)}[1 - p_{(t)}] = M_{(t)} + \int_t^{t+I(t)} \lambda_{(t)}P_{(t)}\,dt,$$

where $M_{[t+I(t)]}$ is the number of such cells present at time $t + I_{(t)}$ and $1 - p_{(t)}$ is the probability that the cells formed at time t will die without further division Substituting from (4) we have

$$P_{(t)}g_{(t)}[1 - p_{(t)}] = P_0 \int_t^{t+I_{(t)}} \lambda_{(t)} \left\{ \exp \int_0^t \frac{\ln [p_{(t)}g_{(t)}]}{I_{(t)}}\, dt \right\} dt,$$

and since $p_{(t)}$, $g_{(t)}$, $I_{(t)}$, and $\lambda_{(t)}$ have been assumed to be constants, we can integrate and find

$$\lambda_{(t)} = \frac{g_{(t)}[1 - p_{(t)}] \ln [p_{(t)}g_{(t)}]}{I_{(t)}[p_{(t)}g_{(t)} - 1]} \tag{7}$$

Equation (6) gives the number of *cells destined to die* which would have been present at time t if there were no cell death If $dM_{(\theta)}/d\theta$ is the rate of formation of such cells at time θ, and $\phi_{(t-\theta,\theta)}$ the probability that these cells will survive up to a later time t, then the number of cells formed after zero time and still present at t is

$$\int_0^t \frac{dM_{(\theta)}}{d\theta} \phi_{(t-\theta,\theta)}\, d\theta$$

If $\eta_{(t)}$ is the probability of survival of the M_0 cells, then the total number of *cells destined to die*, $M_{(t)}$, present at time t is

$$M_{(t)} = M_0\eta_{(t)} + \int_0^t \frac{dM_{(\theta)}}{d\theta} \phi_{(t-\theta,\theta)}\, d\theta,$$

and substituting $dM_{(\theta)}/d\theta$ and $\lambda_{(\theta)}$ from (5) and (7) we obtain

$$M_{(t)} = M_0\eta_{(t)}$$

$$+ P_0 \int_0^t \frac{g_{(\theta)}[1 - p_{(\theta)}] \ln [p_{(\theta)}g_{(\theta)}]}{I_{(\theta)}[p_{(\theta)}g_{(\theta)} - 1]} \left\{ \exp \int_0^\theta \frac{\ln [p_{(\theta)}g_{(\theta)}]}{I_{(\theta)}}\, d\theta \right\} \phi_{(t-\theta,\theta)}\, \theta d \tag{8}$$

To find the total number of cells of both types, $T_{(t)} = P_{(t)} + M_{(t)}$ we combine equations (4) and (8)

B *Nonsteady State with Constant Characteristics*

In this type of cellular proliferation $g_{(\theta)}$, $p_{(\theta)}$, and $I_{(\theta)}$ are constants (i e, independent of θ) and the probability of cell survival $\phi_{(t-\theta,\theta)}$, is independent of the time of cell formation θ Thus they can be represented by g, p, I, and $\phi_{(t-\theta)}$, respectively Equations (4) and (8) then reduce to the following

$$P_{(t)} = P_0 \exp \frac{\ln (pg)}{I}\, t \tag{9}$$

and

$$M_{(t)} = M_0\eta_{(t)} + \frac{g(1 - p) \ln (pg)}{I(pg - 1)} P_0 \int_0^t \left[\exp \frac{\ln (pg)}{I}\, \theta \right] \phi_{(t-\theta)}\, d\theta \tag{10}$$

It would be of interest to know if the ratio $M_{(t)}/P_{(t)}$ changes with time in this case We observe that if τ is the maximum possible life span of the cells, then $\eta_{(t)} = 0$ and for $t - \theta \geq \tau, \phi_{(t-\theta)} = 0$

$$\int_0^t \left[\exp \frac{\ln (pg)}{I}\, \theta \right] \phi_{(t-\theta)}\, d\theta = \int_{t-\tau}^t \left[\exp \frac{\ln (pg)}{I}\, \theta \right] \phi_{(t-\theta)}\, d\theta$$

Thus dividing equation (10) by (9) and utilizing these observations, we obtain for $t \geq \tau$,

$$\frac{M_{(t)}}{P_{(t)}} = \frac{g(1-p)\ln(pg)}{I(pg-1)} \int_{t-\tau}^{t} \left[\exp - \frac{\ln(pg)}{I}(t-\theta)\right] \phi_{(t-\theta)}\, d\theta.$$

Moving the origin of time from zero to $t - \tau$, when t and θ measured from the new origin become τ and $\theta' + t - \tau$, respectively, we have

$$\frac{M_{(t)}}{P_{(t)}} = \frac{g(1-p)\ln(pg)}{I(pg-1)} \int_{0}^{\tau} \left[\exp - \frac{\ln(pg)}{I}(\tau-\theta')\right] \phi_{(\tau-\theta')}\, d\theta' = \rho \qquad (11)$$

This equation shows that the ratio $M_{(t)}/P_{(t)} = \rho$ is independent of t, i e., it remains constant from $t \geq \tau$ The constancy of this ratio will of course be established at a time earlier than τ if the cells were already under identical proliferation conditions prior to zero time (i e , the time of first observation) Equation (11) also demonstrates that both $M_{(t)}$ and $T_{(t)} = P_{(t)} + M_{(t)}$ are following an exponential course, since $M_{(t)}$ follows a parallel course to that of $P_{(t)}$, which according to (9) is exponential in character Thus, exponential proliferation can occur even when some of the cells die, so that the exponential character of the proliferation of cells does not exclude the possibility of concurrent cell death.

A special case that will be considered in this analysis is the case in which the proliferation characteristics are kept constant from at least τ time prior to zero time. We shall refer to this case as *pre-established exponential proliferation.* In this case $M_{(t)}$ and $T_{(t)}$ can be expressed by equations similar to (9) i e ,

$$M_{(t)} = M_0 \exp \frac{\ln(pg)}{I} t \qquad (12)$$

and

$$T_{(t)} = T_0 \exp \frac{\ln(pg)}{I} t, \qquad (13)$$

where M_0 and T_0 are the *cells destined to die* and the total number of cells, respectively, that were present at zero time

If there is no cell death and every cell is destined to divide again, then $p = 1$ and equation (9) reduces to the well known formula

$$P_{(t)} = P_0 \exp \frac{\ln g}{I} t \qquad (14)$$

C. *Steady State*

The steady state is a special case of the previous one in which $p = 1/g.$ Substituting p into equation (9), we find $P_{(t)} = P_0 =$ constant. Since

$$\frac{\ln(pg)}{pg-1} = \frac{\ln 1}{1-1} = \frac{0}{0},$$

we use l'Hospital's rule to evaluate this indeterminate form as follows

$$\lim \frac{\ln(pg)}{pg-1} = \frac{\dfrac{d}{d(pg)}\ln(pg)}{d\,(pg-1)} = \frac{\frac{1}{pg}}{1} = 1.$$

Thus, substituting in (10) we have

$$M_{(t)} = M_0 n_{(t)} + \frac{g-1}{I} P_0 \int_0^t \phi_{(t-\theta)} d\theta, \tag{15}$$

and if τ is the maximum possible life span, then for $t \geq \tau$, $\eta_{(t)} = 0$ and for $t - \theta \geq \tau$, $\phi_{(t-\theta)} = 0$. Thus using the same reasoning as in the derivation of equation (11), we obtain from (15)

$$M_{(t)} = \frac{g-1}{I} P_0 \int_0^\tau \phi_{(x)} dx,$$

which is independent of t and was shown before[8] to be

$$M = P_0 \frac{(g-1)L}{I},$$

where L is the mean life span of the cells.

This will be true for all values of t if this type of proliferation had begun at least τ time prior to zero time. We shall refer to this case as *pre-established steady state*.

II. KINETICS OF ISOTOPE INCORPORATION INTO THE DNA

A. *Generalized Nonsteady State*

1. *Cells destined to divide.* Let $Sm_{(\theta)}$ and $q_{(\theta)}$ be the mean specific activity and the mean DNA content (expressed in terms of the element, an isotope of which is used as tracer) of such cells formed at time θ. Then the amount of isotope entering this compartment of cells during the interval $d\theta$ is $\sigma_{(\theta)} P_{(\theta)} q_{(\theta)} Sm_{(\theta)} d\theta$. If the probability that these cells will not divide prior to a later time t is $\pi_{(t-\theta)}$, and if the isotope is radioactive with a decay constant k, then the fraction of the isotope that was incorporated at time θ and that will be present in these cells at time t is $\sigma_{(\theta)} P_{(\theta)} q_{(\theta)} Sm_{(\theta)} e^{-k(t-\theta)} \pi_{(t-\theta)} d\theta$. Integrating from zero to t we obtain the total amount of isotope present at t

$$\int_0^t \sigma_{(\theta)} P_{(\theta)} q_{(\theta)} Sm_{(\theta)}\, e^{-k(t-\theta)}\, \pi_{(t-\theta)} d\theta$$

Thus, the mean specific activity, $Sr_{(t)}$, of the *cells destined to divide* at time t is

$$Sr_{(t)} = \frac{\int_0^t \sigma_{(\theta)} P_{(\theta)} q_{(\theta)} Sm_{(\theta)}\, e^{-k(t-\theta)} \pi_{(t-\theta)} d\theta}{P_{(t)} q_{(t)}}, \tag{16}$$

where $q_{(t)}$ is the mean DNA content of these cells at time t. Following similar reasoning we can deduce an equation giving $q_{(t)}$,

$$q_{(t)} = \frac{\int_0^t \sigma_{(\theta)} P_{(\theta)} q_{(\theta)} \pi_{(t-\theta)} d\theta}{P_{(t)}} \tag{17}$$

Substituting $q_{(t)}$ and $\sigma_{(\theta)}$ and $P_{(\theta)}$ from equations (3) and (4) into (16), we obtain

$$Sr_{(t)} = \frac{\int_0^t \frac{q_{(\theta)} \ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}} \left\{ \exp \int_0^\theta \frac{\ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}} \, d\theta \right\} S_{n(\theta)} \, e^{-k(t-\theta)} \pi_{(t-\theta)} \, d\theta}{\int_0^t \frac{q_{(\theta)} \ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}} \left\{ \exp \int_0^\theta \frac{\ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}} \, d\theta \right\} \pi_{(t-\theta)} \, d\theta} \tag{18}$$

In turn we can derive an equation for $S_{m(t)}$ as follows If $dP_{(\theta)}$ is the number of *cells destined to divide* which are formed at time θ during the interval $d\theta$ and if they have a probability $\pi_{(t-\theta)}$ that they will not divide prior to a later time, t, and a probability $1 - \pi_{(t-\theta)}$ that they will divide prior to time t then the probability that they will divide at an age $t - \theta$ (i e the rate of cell division at time t) is

$$\frac{\partial[1 - \pi_{(t-\theta)}]}{\partial(t - \theta)} = -\frac{\partial \pi_{(t-\theta)}}{\partial(t - \theta)}$$

and the number of these cells which will divide at time t during the interval dt is

$$dP_{(\theta)} \left[-\frac{\partial \pi_{(t-\theta)}}{\partial(t - \theta)} \right] dt$$

If $q_{(t)}$ is the mean DNA content of the cells produced at time t and *destined to divide* and $q'_{(t)}$ that of those *destined to die*, then each of the *cells destined to divide* will retain on the average

$$q_{(\theta)} \frac{q_{(t)}}{g_{(t)}\{p_{(t)}q_{(t)} + [1 - p_{(t)}]q'_{(t)}\}} = \frac{q_{(\theta)}}{h_{(t)}} \tag{19}$$

of the original DNA and acquire $q_{(t)} - q_{(\theta)}/h_{(t)} - u_{(t)}q_{(t)}$ of newly formed DNA The function $u_{(t)}$ represents a proportionality factor which, when multiplied by the amount of DNA per cell, $q_{(t)}$, gives the fraction of the DNA $u_{(t)}q_{(t)}$ which originates from dead cells and which is reutilized at time t without prior degradation Therefore, the amount of isotope present in these cells is

$$dP_{(\theta)} \left[-\frac{\partial \pi_{(t-\theta)}}{\partial(t - \theta)} \right] \left\{ \frac{q_{(\theta)}}{h_{(t)}} S_{m(\theta)} e^{-k(t-\theta)} + \left[q_{(t)} - \frac{q_{(\theta)}}{h_{(t)}} - u_{(t)}q_{(t)} \right] S_{(t)} + u_{(t)}q_{(t)}S_{u(t)} \right\} dt,$$

where $S_{(t)}$ is the specific activity of the DNA precursor, $S_{u(t)}$ is the specific activity of the reutilized DNA, and $e^{-k(t-\theta)}$ corrects for the physical decay of the isotope of the original DNA Integrating from zero to t, we find the total amount of isotope, $dR^*_{(t)}$, that will be present in the cells which entered mitosis at time t during the interval dt

$$dR^*_{(t)} = \left\langle \int_0^t -\frac{\partial \pi_{(t-\theta)}}{\partial(t - \theta)} \left\{ \frac{q_{(\theta)}}{h_{(t)}} S_{m(\theta)} e^{-k(t-\theta)} + \left[q_{(t)} - \frac{q_{(\theta)}}{h_{(t)}} - u_{(t)}q_{(t)} \right] S_{(t)} + u_{(t)} q_{(t)} S_{u(t)} \right\} dP_{(\theta)} \right\rangle d$$

Therefore,

$$Sm_{(t)} = \frac{dR^*_{(t)}}{dR_{(t)}}$$

$$= \frac{\left\langle \int_0^t -\frac{\partial \pi_{(t-\theta,\,\theta)}}{\partial(t-\theta)} \left\{ \frac{q_{(\theta)}}{h_{(t)}}\, Sm_{(\theta)}\, e^{-k(t-\theta)} + \left[q_{(t)} - \frac{q_{(\theta)}}{h_{(t)}} - u_{(t)} q_{(t)} \right] S_{(t)} + u_{(t)} q_{(t)} Su_{(t)} \right\} dP_{(\theta)} \right\rangle dt}{dP_{(t)} q_{(t)}},$$

where $dR_{(t)} = dP_{(t)} q_{(t)}$ is the amount of DNA present in these cells Substituting $dP_{(t)}$ from (1) and taking $\sigma_{(t)}$ and $P_{(t)}$ from (3) and (4), we obtain

$$Sm_{(t)} = \frac{\int_0^t \left\{ \frac{q_{(\theta)}}{h_{(t)}}\, Sm_{(\theta)}\, e^{-k(t-\theta)} + \left[q_{(t)} - \frac{q_{(\theta)}}{h_{(t)}} - u_{(t)} q_{(t)} \right] S_{(t)} + u_{(t)} q_{(t)} Su_{(t)} \right\} \left[-\frac{\partial \pi_{(t-\theta,\,\theta)}}{\partial(t-\theta)} \right] \frac{\ln\,[p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}} \left\{ \exp \int_0^\theta \frac{\ln\,[p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}}\, d\theta \right\} d\theta}{\frac{q_{(t)} \ln\,[p_{(t)} g_{(t)}]}{I_{(t)}} \exp \int_0^t \frac{\ln\,[p_{(t)} g_{(t)}]}{I_{(t)}}\, dt} \qquad (20)$$

Assuming that the DNA of the dead cells is randomly reutilized, and disregarding the lag of time between cell death and DNA reutilization, we can derive an equation for $Su_{(t)}$ If $\phi_{(t-\theta,\,\theta)}$ is the probability that cells formed at time θ will survive at least up to a later time t, then the probability that they will die prior to time t is $1 - \phi_{(t-\theta,\,\theta)}$ and the probability that they will die at an age $t - \theta$ is

$$\frac{\partial[1 - \phi_{(t-\theta,\,\theta)}]}{\partial(t-\theta)} = -\frac{\partial \phi_{(t-\theta,\,\theta)}}{\partial(t-\theta)}$$

Thus, if the number of *cells destined to die* which are formed at time θ during the interval $d\theta$ is $dM_{(\theta)}$, the number of these cells which will die at time t during the interval dt is

$$dM_{(\theta)} \left[-\frac{\partial \phi_{(t-\theta,\,\theta)}}{\partial(t-\theta)} \right] dt$$

If $q'_{(\theta)}$ is the mean DNA content of each of these cells, then their total DNA content is

$$dM_{(\theta)} q'_{(\theta)} \left[-\frac{\partial \phi_{(t-\theta,\,\theta)}}{\partial(t-\theta)} \right] dt,$$

and the amount of isotope in them is

$$dM_{(\theta)} q'_{(\theta)} \left[-\frac{\partial \phi_{(t-\theta,\,\theta)}}{\partial(t-\theta)} \right] Sm_{(\theta)}\, e^{-k(t-\theta)} dt,$$

where $e^{-k(t-\theta)}$ corrects for the physical decay of the isotope Integrating these two expressions from zero to t, we obtain the total DNA and the total isotope contained in the DNA of the cells which die at time t Therefore the specific activity, $Su_{(t)}$, of these cells is

$$Su_{(t)} = \frac{\int_0^t \frac{\partial \phi_{(t-\theta,\theta)}}{\partial (t-\theta)} q'_{(\theta)} Sm_{(\theta)} e^{-k(t-\theta)} dM_{(\theta)}}{\int_0^t \frac{\partial \phi_{(t-\theta,\theta)}}{\partial (t-\theta)} q'_{(\theta)} dM_{(\theta)}}$$

Substituting $dM_{(\theta)}$ from (5) and taking $\lambda_{(\theta)}$ from (7), we obtain

$$Su_{(t)} = \frac{\int_0^t \frac{g_{(\theta)} q'_{(\theta)} [1 - p_{(\theta)}] \ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)} [p_{(\theta)} g_{(\theta)} - 1]} \left\{ \exp \int_0^\theta \frac{\ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}} d\theta \right\} Sm_{(\theta)} \frac{\partial \phi_{(t-\theta,\theta)}}{\partial (t-\theta)} d\theta}{\int_0^t \frac{g_{(\theta)} q'_{(\theta)} [1 - p_{(\theta)}] \ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)} [p_{(\theta)} g_{(\theta)} - 1]} \left\{ \exp \int_0^\theta \frac{\ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}} d\theta \right\} \frac{\partial \phi_{(t-\theta,\theta)}}{\partial (t-\theta)} d\theta} \qquad (21)$$

If no reutilization of intact DNA molecules occurs then $u_{(t)} \equiv 0$ and equation (20) reduces to

$$Sm_{(t)} = \frac{\int_0^t \left\{ \frac{q_{(\theta)}}{h_{(t)}} Sm_{(\theta)} e^{-k(t-\theta)} + \left[q_{(t)} - \frac{q_{(\theta)}}{h_{(t)}} \right] S_{(t)} \right\} \left[-\frac{\partial \pi_{(t-\theta,\theta)}}{\partial (t-\theta)} \right] \frac{\ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}} \left\{ \exp \int_0^\theta \frac{\ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}} d\theta \right\} d\theta}{\frac{q_{(t)} \ln [p_{(t)} g_{(t)}]}{I_{(t)}} \exp \int_0^t \frac{\ln [p_{(t)} g_{(t)}]}{I_{(t)}} dt} \qquad (22)$$

2 *Cells destined to die* The rate of entrance of DNA into these cells is

$$\frac{dM_{(t)}}{dt} q'_{(t)} = \lambda_{(t)} q'_{(t)} P_{(t)},$$

where $q'_{(t)}$ is the mean DNA content of such cells which are formed at time t. The specific activity of their DNA is $Sm_{(t)}$, therefore the amount of isotope that is present in such cells which are formed at time θ during the interval $d\theta$ is

$$\lambda_{(\theta)} q'_{(\theta)} P_{(\theta)} Sm_{(\theta)} d\theta$$

If $\phi_{(t-\theta,\theta)}$ is the probability that these cells will survive at least up to a later time t, then the fraction of this isotope that would still be present in this compartment at time t is

$$\lambda_{(\theta)} q'_{(\theta)} P_{(\theta)} Sm_{(\theta)} e^{-k(t-\theta)} \phi_{(t-\theta,\theta)} d\theta,$$

where $e^{-k(t-\theta)}$ corrects for the physical decay. Integrating from zero to t we obtain the total amount of isotope present in this compartment at time t, which when divided by the total DNA gives the specific activity $Sd_{(t)}$,

$$Sd_{(t)} = \frac{\int_0^t \lambda_{(\theta)} q'_{(\theta)} P_{(\theta)} Sm_{(\theta)} e^{-k(t-\theta)} \phi_{(t-\theta,\theta)} d\theta}{M_{(t)} \bar{q}'_{(t)}}, \qquad (23)$$

where $\bar{q}'_{(t)}$ is the mean DNA content per cell at time t in this compartment and is given by the equation

$$q'_{(t)} = \frac{q'_{0(t)} M_0 n_{(t)} + \int_0^t \lambda_{(\theta)} q'_{(\theta)} P_{(\theta)} \phi_{(t-\theta,\theta)}\, d\theta}{M_{(t)}}, \tag{24}$$

where $q'_{0(t)}$ is the mean DNA content at time t of the *cells destined to divide* which were present at zero time and are still present at time t Substituting $q'_{(t)}$, $\lambda_{(\theta)}$, $P_{(\theta)}$, and $M_{(t)}$ from equations (24), (7), (4), and (8) into (23) we have

$$Sd_{(t)} = \frac{P_o \int_0^t \frac{g_{(\theta)}[1 - p_{(\theta)}] \ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}[p_{(\theta)} g_{(\theta)} - 1]} q'_{(\theta)} \left\{\exp \int_0^\theta \frac{\ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}}\, d\theta\right\} Sm_{(\theta)}\, e^{-k(t-\theta)} \phi_{(t-\theta,\theta)}\, d\theta}{q'_{o(t)} M_o n_{(t)} + P_o \int_0^t \frac{g_{(\theta)}[1 - p_{(\theta)}] \ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}[p_{(\theta)} g_{(\theta)} - 1]} q'_{(\theta)} \left\{\exp \int_0^\theta \frac{\ln [p_{(\theta)} g_{(\theta)}]}{I_{(\theta)}}\, d\theta\right\} \phi_{(t-\theta,\theta)}\, d\theta} \tag{25}$$

3 *Both cell types combined* The specific activity $Sq_{(t)}$ of the DNA of the combined cell population, $T_{(t)} = P_{(t)} + M_{(t)}$, can be obtained by multiplying the amount of DNA present in each of the two cell types by its respective specific activity, and dividing the sum of the products by the total DNA, i e ,

$$Sq_{(t)} = \frac{P_{(t)} q_{(t)} Sr_{(t)} + M_{(t)} q'_{(t)} Sd_{(t)}}{P_{(t)} q_{(t)} + M_{(t)} q'_{(t)}} = \frac{C_{(t)} Sr_{(t)} + Sd_{(t)}}{C_{(t)} + 1}, \tag{26}$$

where

$$C_{(t)} = \frac{P_{(t)} q_{(t)}}{M_{(t)} q'_{(t)}}$$

$P_{(t)}$, $M_{(t)}$, $q_{(t)}$, $q'_{(t)}$, $Sr_{(t)}$, and $Sd_{(t)}$ are taken from equations (4), (8), (17), (24), (18), and (25)

A second approach to this problem is to consider the mixed population of cells without subdividing them into two types Thus, if $dV_{(\theta)}$ is the number of new cells formed at time θ, $qn_{(\theta)}$ their mean DNA content, and $\Phi_{(t-\theta,\theta)}$ the probability that they will still be present at a later time t (i e , that they will neither divide nor die prior to time t), then following the same reasoning as that used in the derivation of equation (16), we find

$$Sq_{(t)} = \frac{\int_0^t Sm_{(\theta)}\, dV_{(\theta)} qn_{(\theta)} \Phi_{(t-\theta,\theta)}\, e^{-k(t-\theta)}}{T_{(t)} qn_{(t)}}, \tag{27}$$

where $qn_{(t)}$ is the mean DNA content per cell at time t and is given by the equation

$$qn_{(t)} = \frac{\int_0^t dV_{(\theta)} qn_{(\theta)} \Phi_{(t-\theta,\theta)}}{T_{(t)}}$$

$qn_{(\theta)}$ can be expressed in terms of $q_{(\theta)}$, $q'_{(\theta)}$, and $p_{(\theta)}$ as follows

$$qn_{(\theta)} = p_{(\theta)} q_{(\theta)} + [1 - p_{(\theta)}] q'_{(\theta)}$$

We observe that $dN_{(t)} = dP_{(t)} + dM_{(t)}$, and substituting from (1), (4), (5), and (7), we have

$$dN_{(t)} = \frac{[g_{(t)} - 1] \ln [p_{(t)}g_{(t)}]}{I_{(t)}[p_{(t)}g_{(t)} - 1]} P_o \left\{ \exp \int_0^t \frac{\ln [p_{(t)}g_{(t)}]}{I_{(t)}} dt \right.$$

Substituting $\bar{q}_{n(t)}$ and $dN_{(t)}$ into (27) we obtain

$$S_{q(t)} = \frac{\displaystyle\int_0^t \frac{[g_{(\theta)} - 1] \ln [p_{(\theta)}g_{(\theta)}]}{I_{(\theta)}[p_{(\theta)}g_{(\theta)} - 1]} q_{n(\theta)} \left\{ \exp \int_0^\theta \frac{\ln [p_{(\theta)}g_{(\theta)}]}{I_{(\theta)}} d\theta \right\} S_{m(\theta)} e^{-k(t-\theta)} \Phi_{(t-\theta,\theta)} d\theta}{\displaystyle\int_0^t \frac{[g_{(\theta)} - 1] \ln [p_{(\theta)}g_{(\theta)}]}{I_{(\theta)}[p_{(\theta)}g_{(\theta)} - 1]} q_{n(\theta)} \left\{ \exp \int_0^\theta \frac{\ln [p_{(\theta)}g_{(\theta)}]}{I_{(\theta)}} d\theta \right\} \Phi_{(t-\theta,\theta)} d\theta} \tag{28}$$

Equations (18), (20), (21), (22), (25), (26), and (28) show that $\pi_{(t-\theta,\theta)}$, $\phi_{(t-\theta,\theta)}$, or $\Phi_{(t-\theta,\theta)}$ cannot be obtained solely from data on the isotope incorporation into the DNA of the two types of cells, since these equations can not be solved unless the functions $g_{(t)}$, $I_{(t)}$, $p_{(t)}$, $q_{(t)}$, $q'_{(t)}$, $q'_{o(t)}$, $\eta_{(t)}$, and $u_{(t)}$ are known, and then they can be solved only through a change of variables by placing $t - \theta = x$, which is permissible only if the probabilities of survival and division are independent of the time of the formation of the cell

B *Nonsteady State with Constant Characteristics*

The previous equations become much simpler if $g_{(t)} \equiv g$, $p_{(t)} \equiv p$ $I_{(t)} \equiv I$, $q_{(t)} \equiv \bar{q}_{(t)} \equiv q$, $q'_{o(t)} \equiv q'_{(t)} \equiv \bar{q}'_{(t)} \equiv q'$, and $u_{(t)} \equiv u$, i e, constants independent of t, and if $\pi_{(t-\theta,\theta)}$ and $\phi_{(t-\theta,\theta)}$ are identical for all generations of cells so that we can change variables by placing $t - \theta = x$ and $d\theta = -dx$

1 *Cells destined to divide* Substituting these constants and changing variables as indicated above, we obtain from equation (18)

$$\begin{aligned} S_{r(t)} \int_0^t \left[\exp - \frac{\ln (pg)}{I} x \right] \pi_{(x)} dx \\ = \int_0^t \left\{ \exp - \left[k + \frac{\ln (pg)}{I} \right] x \right\} S_{m(t-x)} \pi_{(x)} dx \end{aligned} \tag{29}$$

Equation (29) is simplified for values of $t \geq I_m$ where I_m is the maximum generation time Since $\pi_{(x)} = 0$ for $x \geq I_m$, we have

$$\int_0^t \left[\exp - \frac{\ln (pg)}{I} x \right] \pi_{(x)} dx = \int_0^{I_m} \left[\exp - \frac{\ln (pg)}{I} x \right] \pi_{(x)} dx = \frac{1}{c} = \text{constant}$$

Thus, equation (29) can be written in the form

$$S_{r(t)} = c \int_0^t \left\{ \exp - \left[k + \frac{\ln (pg)}{I} \right] x \right\} S_{m(t-x)} \pi_{(x)} dx, \tag{30}$$

which is a Volterra equation of the first kind and can be solved for $\pi_{(x)}$ by the usual methods, provided that $S_{r(t)}$ and $S_{m(t)}$ are known functions.

An alternative way of solving equation (30) is possible if $S_{m(t-x)}$ can be written in the form

$$Sm_{(t-x)} = \sum_{i=1}^{z} \beta_{i(t)}\gamma_{i(x)} \tag{31}$$

Equation (30) then becomes

$$Sr_{(t)} = c \sum_{i=1}^{z} \beta_{i(t)} \int_0^t \left\{\exp - \left[k + \frac{\ln (pg)}{I}\right] x\right\} \gamma_{i(x)}\pi_{(x)} dx, \tag{32}$$

which can be transformed into a linear differential equation through z successive differentiations and back substitutions to eliminate the integrals in a manner similar to that used previously[8] If the cell proliferation had proceeded under identical conditions for at least Im time prior to zero time, then equations (30) and (32) are valid throughout the experimental period The constants c and $[\ln (pg)]/I$ may be evaluated by utilizing the observation that at $t = 0$ we have $\pi_{(0)} = 1$ and $[d'\pi_{(t)}/dt']_{t=0} = 0$ This is because a definite interval of time must lapse between two successive mitoses of any cell Similarly at $t \geq Im$, we have $\pi_{(t)} = 0$ and $[d'\pi_{(t)}/dt']_{t \geq Im} = 0$ If the characteristic constants of cell proliferation prior to and after zero time are not identical, then we may proceed as follows Differentiating equation (29) in respect to t, we have

$$\begin{aligned} \frac{dSr_{(t)}}{dt} \int_0^t \left[\exp - \frac{\ln (pg)}{I} x\right] \pi_{(x)} dx + Sr_{(t)} \left[\exp - \frac{\ln (pg)}{I} t\right] \pi_{(t)} \\ = \int_0^t \frac{\partial}{\partial t} Sm_{(t-x)} \left\{\exp - \left[k + \frac{\ln (pg)}{I}\right] x\right\} \pi_{(x)} dx \\ + \left\{\exp - \left[k + \frac{\ln (pg)}{I}\right] t\right\} Sm_{(t-t)}\pi_{(t)}, \end{aligned}$$

and since $Sm_{(t-t)} = Sm_{(0)} = 0$ we can write this equation in the following form

$$\pi_{(t)} = \int_0^t \frac{\left[\frac{\partial}{\partial t} Sm_{(t-x)}\, e^{-kx} - \frac{dSr_{(t)}}{dt}\right]}{Sr_{(t)}} \left[\exp \frac{\ln (pg)}{I} (t - x)\right] \pi_{(x)} dx \tag{33}$$

This is a homogeneous integral equation the general solution of which, $\pi_{(t)} \equiv 0$, is meaningless[2] If, however, $Sr_{(t)}$ and $Sm_{(t)}$ can be determined experimentally and then equations are found to describe them, it may be possible to find the *eigenvalues* and the *eigenfunctions* of this equation and then to find an acceptable general solution The evaluation of the constant $[\ln (pg)]/I$ can be done from the equation of $\pi_{(t)}$ and/or its derivatives by observing that if $t = 0$, $\pi_{(0)} = 1$, and $[d'\pi_{(t)}/dt']_{t=0} = 0$, and if $t \geq Im$, $\pi_{(t)} = 0$, and $[d'\pi_{(t)}/dt']_{t \geq Im} = 0$

An alternative way of solving the equation (29) is possible if $Sm_{(t-x)}$ is of the form given by (31) In this case, we may proceed as in (32)

Similarly, equations (20), (21), and (22) reduce, respectively, to

$$\begin{aligned} Sm_{(t)} = \frac{1}{h} \int_0^t \{Sm_{(t-x)}\, e^{-kx} + [(1 - u)h - 1]S_{(t)} \\ + huSu_{(t)}\} \left[\exp - \frac{\ln (pg)}{I} x\right] \left[-\frac{d\pi_{(x)}}{dx}\right] dx, \end{aligned} \tag{34}$$

$$S_{u(t)} = \frac{\int_0^t S_{m(t-x)} \left\{ \exp - \left[k + \frac{\ln (pg)}{I} \right] x \right\} \frac{d\phi_{(x)}}{dx}\, dx}{\int_0^t \left[\exp - \frac{\ln (pg)}{I}\, x \right] \frac{d\phi_{(x)}}{dx}\, dx}, \tag{35}$$

and

$$S_{m(t)} = \frac{1}{h} \int_0^t [S_{m(t-x)}\, e^{-kx} + (h - 1) S_{(t)}] \left[\exp - \frac{\ln (pg)}{I}\, x \right] \left[- \frac{d\pi_{(x)}}{dx} \right] dx, \tag{36}$$

where $1/h = q/\{[pq + (1 - pq')]g\}$ is obtained from (19). Equation (36) is a Volterra equation of the first kind and can be solved by the usual methods³ to yield the function $-(d\pi_{(x)}/dx)$, i.e., the rate of cell division, provided that $S_{m(t)}$ and $S_{(t)}$ can be obtained experimentally and equations can be found to describe them. It is also possible to transform this equation into a linear differential equation, as in the case of equation (30), if the equation for $S_{m(t-x)}$ is of the form of (31) and if we let $S_{(t)} = \sum_{i=1}^{7} \alpha\, e^{r_i t}$. The constants $1/h$ and $[\ln (pg)]/I$ may be evaluated by observing that at $t = 0$, $\pi_{(0)} = 1$, $[d^i\pi_{(t)}/dt^i]_{t=0} = 0$, and at $t \geq Im$, $\pi_{(t)} = 0$ and $[d^i\pi_{(t)}/dt^i]_{t \geq Im} = 0$. Integration of $d\pi_{(x)}\, dx$ will yield the function $\pi_{(x)}$.

2. *Cells destined to die.* Introducing the same substitutions and change of variables into equation (25) we obtain

$$S_{d(t)} = \frac{\frac{g(1 - p) \ln (pg)}{I(pg - 1)} P_o \left[\exp \frac{\ln (pg)}{I}\, t \right] \int_0^t \left\{ \exp - \left[k + \frac{\ln (pg)}{I} \right] x \right\} S_{m(t-x)} \phi_{(x)}\, dx}{M_o \eta_{(t)} + \frac{g(1 - p) \ln (pg)}{I(pg - 1)} P_o \left[\exp \frac{\ln (pg)}{I}\, t \right] \int_0^t \left[\exp - \frac{\ln (pg)}{I}\, x \right] \phi_{(x)} dx} \tag{37}$$

When $t \geq \tau$ then $\eta_{(t)} = 0$

$$\therefore S_{d(t)} \int_0^t \left[\exp - \frac{\ln (pg)}{I}\, x \right] \phi_{(x)} dx = \int_0^t \left\{ \exp - \left[k + \frac{\ln (pg)}{I} \right] x \right\} S_{m(t-x)} \phi_{(x)} dx.$$

It has been shown by the derivation of (11) that the integral

$$\int_0^t \left[\exp - (\ln (pg))/Ix \right] \phi_{(x)} dx$$

is constant and equal to $[I(pg - 1)\rho]/[g(1 - p) \ln (pg)]$. Substituting in the above equation, we have

$$S_{d(t)} = \frac{g(1 - p) \ln (pg)}{I(pg - 1)\rho} \int_0^t \left\{ \exp - \left[k + \frac{\ln (pg)}{I} \right] x \right\} S_{m(t-x)} \phi_{(x)} dx \tag{38}$$

This equation is similar to (30) and may be solved by the same methods to yield $\phi_{(x)}$, if $S_{d(t)}$ and $S_{m(t-x)}$ are known functions. It should be noted, however, that in order to apply this equation, we should be able to follow the disappearance of the M_o cells If the isotope is radioactive and if it is possible to elute all the non DNA isotope from the cells, then the disappearance of the M_o cells may be followed with the aid of radioautographs In the case of *pre-established exponential proliferation*, equation (38) is valid throughout the experiment The same alternative solution of (38) is possible as it is in (30), if $Sm_{(t-x)}$ can be written in the form given by (31) The constants $[g(1 - p) \ln (pg)]/[I(pg - 1)\rho]$ and $[\ln (pg)]/I$ may be evaluated by observing that at $t = 0$ we have $\phi_{(0)} = 1$ and $[d\phi_{(t)}/dt']_{t=0} = 0$, and at $t \geq \tau, \phi_{(t)} = 0$ and $[d\phi_{(t)}/dt']_{t\geq\tau} = 0$

3 *Both cell types combined* We have already shown that the ratio $P_{(t)}$ $M_{(t)}$ is constant if the proliferation is of the pre-established exponential type or if $t \geq \tau$ Thus, substituting from (11) into equation (26) we obtain

$$Sq_{(t)} = \frac{qSr_{(t)} + \rho q' Sd_{(t)}}{q + \rho q'}. \tag{39}$$

We may then take $Sr_{(t)}$ and $Sd_{(t)}$ from (30) and (38) when we have

$$Sq_{(t)} = \frac{1}{q + \rho q'} \int_0^t \left\{\exp - \left[\lambda + \frac{\ln (pg)}{I}\right]x\right\} Sm_{(t-x)} \left[qc\pi_{(x)} + \frac{q'g(1 - p) \ln (pg)}{I(pg - 1)} \phi_{(x)}\right] dx \tag{40}$$

Equation (28) is also simplified to the following

$$Sq_{(t)} \int_0^t \left[\exp - \frac{\ln (pg)}{I} x\right] \Phi_{(x)} dx = \int_0^t \left\{\exp - \left[\lambda + \frac{\ln (pg)}{I}\right]x\right\} Sm_{(t-x)} \Phi_{(x)}\, dx, \tag{41}$$

and by using the same reasoning as in the derivation of (30) we find

$$Sq_{(t)} = c' \int_0^t \left\{\exp - \left[\lambda + \frac{\ln (pg)}{I}\right]x\right\} Sm_{(t-x)} \Phi_{(x)}\, dx \tag{42}$$

where c' is a constant Equations (41) and (42) are similar to (29) and (30), respectively, and can be solved by similar procedures to yield $\Phi_{(x)}$ A comparison of equation (40) and (42) brings out the following relationships

$$c' = \frac{1}{q + \rho q'} \tag{43}$$

and

$$\Phi_{(x)} = cq\pi_{(x)} + \frac{q'g(1 - p) \ln (pg)}{I(pg - 1)} \tag{44}$$

C *Steady State*

This case is the same as the preceding one, with the exception that $p = 1/g$ We can thus place $pg = 1$ and $\ln (pg) = 0$ into the equations of the previous section to obtain the equations for the steady state

1 *Cells destined to divide* Equation (29) becomes

$$Sr_{(t)} \int_0^t \pi_{(x)}\, dx = \int_0^t e^{-kx} Sm_{(t-x)} \pi_{(x)}\, dx \tag{45}$$

If $t \geq I_m$, or if this type of proliferation commenced at least I_m time prior to zero time, then since $[\pi_{(x)}]_{t \geq I_m} = 0$, we have

$$\int_0^t \pi_{(x)}\, dx = \int_0^{I_m} \pi_{(x)}\, dx = \pi_{(x)} x \Big|_0^{I_m} + \int_0^{I_m} x d[1 - \pi_{(x)}] = I$$

$$\therefore\ Sr_{(t)} = \frac{1}{I} \int_0^t e^{-kx} Sm_{(t-x)} \pi_{(x)}\, dx \tag{46}$$

These two equations are similar to (29) and (30), respectively, and can be solved by the same technics to give $\pi_{(x)}$ if $Sr_{(t)}$ and $Sm_{(t)}$ are known

Similarly, equations (34), (35), and (36) reduce to the similar equations

$$Sm_{(t)} = \frac{1}{h} \int_0^t \{Sm_{(t-x)}\, e^{-kx} + [(1 - u)h - 1]S_{(t)} + huSu_{(t)}\} \left[-\frac{d\pi_{(x)}}{dx}\right] dx, \tag{47}$$

$$Su_{(t)} = \frac{\int_0^t Sm_{(t-x)}\, e^{-kx} \frac{d\phi_{(x)}}{dx}\, dx}{\phi_{(t)} - 1}, \tag{48}$$

and

$$Sm_{(t)} = \frac{1}{h} \int_0^t [Sm_{(t-x)}\, e^{-kx} + (h - 1)S_{(t)}] \left[-\frac{d\pi_{(x)}}{dx}\right] dx \tag{49}$$

which can be solved by the same technics to yield $-(d\pi_{(x)}/dx)$, if $Sm_{(t)}$ is known In this case $1/h = q/[q + (g - 1)q']$

2 *Cells destined to die.* We have already shown that $[\ln (pg)]/(pg - 1) = 1$ when $p = 1/g$ Therefore, substituting into equation (37) we find

$$Sd_{(t)} = \frac{\frac{g-1}{I} P_o \int_0^t e^{-kx} Sm_{(t-x)} \phi_{(x)}\, dx}{Mon_{(t)} + \frac{g-1}{I} P_o \int_0^t \phi_{(x)}\, dx}. \tag{50}$$

When $t \geq \tau$, then $\eta_{(t)} = 0$ and $\int_0^t \phi_{(x)} dx = L$,[8] therefore

$$Sd_{(t)} = \frac{1}{L} \int_0^t e^{-kx} Sm_{(t-x)} \phi_{(x)}\, dx \tag{51}$$

We also note that in the case of a *pre-established steady state*, equation (51) is valid for all values of t This equation is similar to (46) and can be solved to give the probability of survival $\phi_{(x)}$ if the functions $Sd_{(t)}$ and $Sm_{(t)}$ are known

3 *Both cell types combined* It has been shown[8] that in the steady state we have

$$\frac{P}{M} = \frac{I}{(g-1)L}$$

Substituting in equation (39), we obtain

$$S_{q(t)} = \frac{IqS_{r(t)} + (g-1)Lq'S_{d(t)}}{qI + (g-1)q'L}$$

This equation is valid for values of $t \geq \tau$, and in the case of a *pre-established steady state*, it is valid for all values of t. Substituting $S_{r(t)}$ and $S_{d(t)}$ from (46) and (51), we have

$$S_{q(t)} = \frac{\int_0^t e^{-kx} S_{m(t-x)}[q\pi_{(x)} + (g-1)q'\phi_{(x)}]\,dx}{qI + (g-1)q'L} \tag{52}$$

Similarly, equations (41) and (42) become

$$S_{q(t)} \int_0^t \Phi_{(x)}\,dx = \int_0^t e^{-kx} S_{m(t-x)}\Phi_{(x)}\,dx \tag{53}$$

and

$$S_{q(t)} = c' \int_0^t e^{-kx} S_{m(t-x)}\Phi_{(x)}\,dx \tag{54}$$

These are similar to (41) and (42) and can be solved by the same technics to give $\Phi_{(x)}$ if $S_{q(t)}$ and $S_{m(t)}$ are known. A comparison of (52) and (54) reveals that

$$c' = \frac{1}{qI + (g-1)q'L} \tag{55}$$

and

$$\Phi_{(x)} = q\pi_{(x)} + (g-1)q'\phi_{(x)} \tag{56}$$

D. *Determination of $S_{m(t)}$*

The analysis up to this point relies entirely on knowledge of the $S_{m(t)}$. Information on $S_{m(t)}$ may be obtained with the aid of radioautographs of cells, provided that all the non-DNA isotope can be eliminated. If, then, the mean specific activity $S_{q(t)}$ of the DNA is known and if the mean number of silver grains per cell, $G1_{(t)}$, and per mitosis, $G2_{(t)}$, can be determined from the radioautographs, we have

$$S_{q(t)} = \mu \frac{G1_{(t)}}{qp + q'(1-p)} \tag{57}$$

and

$$S_{m(t)} = \mu \frac{G2_{(t)}}{g[qp + q'(1-p)]}, \tag{58}$$

where μ is a proportionality constant, $qp + q'(1-p)$ the mean DNA content per cell (*vide supra*), and $g[qp + q'(1-p)]$ the mean DNA content per mitosis (note that the DNA of cells found in mitosis should have increased to an amount necessary to give *gp cells destined to divide* and $g(1 - p)$ *cells destined to die*) Combining the above equations, we find

$$S_{m(t)} = \frac{S_{q(t)} G2_{(t)}}{g G1_{(t)}} \tag{59}$$

It should be noted, however, that this method gives only the mean specific activity of the cells formed during the mitotic interval if every cell found in mitosis is included Ideally, to obtain the DNA specific activity of cells formed at time t, one should use only cells in an identical stage of mitosis

This method can be used also to determine the specific activity of the DNA of cells which are mixed with other types of cells. Thus, if the DNA specific activity, the mean number of grams per cell and the mean DNA content per cell of a mixed population of cells is represented by $S1_{(t)}$ $G1_{(t)}$ and q_1 respectively, and those of one type of cell of the mixture by $S2_{(t)}$ $G2_{(t)}$ and q_2 we have

$$S2_{(t)} = S1_{(t)} \frac{G2_{(t)}\, q_1}{G1_{(t)}\, q_2} \tag{60}$$

Equation (60) can be used to determine for example the DNA specific activity of each of the types of leukocytes present in a mixed sample

E *Approximate Methods If $S_{m(t)}$ is Unknown*

An approximate method which does not require the determination of $S_{m(t)}$ may be followed in the cases of the *pre established exponential proliferation* and *pre-established steady state*, if it can be reasonably assumed that the generation time of every cell is practically the same and that there is no reutilization of intact DNA molecules from dead cells In this case, the function $\pi_{(x)}$ becomes $\pi_{(x)} = 0$ for $x \geq I$ and

$$\pi_{(x)} = 1 \quad \text{for} \quad 0 < x < I$$

Thus equation (30) reduces to

$$S_{r(t)} = c \int_{t-I}^{t} \left\{ \exp - \left[\lambda + \frac{\ln (pg)}{I} \right] x \right\} S_{m(t-x)}\, dx \tag{61}$$

Under these conditions $S_{r(t)}$ is given by the previously derived equation[8]

$$S_{r(t)} = S_0 \frac{\ln h}{I} \sum_{i=1}^{\nu} \frac{\alpha}{\lambda + r_i + \dfrac{\ln h}{I}} \left[e^{r_i t} - \exp - \left(\lambda + \frac{\ln h}{I} \right) t \right] \tag{62}$$

Changing variables in (61) by letting $x = t - \theta$, then $dx = -d\theta$, and equating with (62) we obtain

$$c \int_{t-I}^{t} \left\{ \exp \left[\lambda + \frac{\ln pg}{I} \right] \theta \right\} S_{m(\theta)}\, d\theta = S_0 \frac{\ln h}{I}$$

$$\sum_{i=1}^{\nu} \frac{\alpha_i}{\lambda + r_i + \dfrac{\ln h}{I}} \left\langle \left\{ \exp \left[\lambda + r_i + \frac{\ln (pg)}{I} \right] t \right\} - \exp \frac{\ln \dfrac{pg}{h}}{I} t \right\rangle$$

Differentiating in respect to t we have

$$c\left\{\exp\left[k+\frac{\ln pg}{I}\right]t\right\}\left[Sm_{(t)}-Sm_{(t-I)}\,e^{-kI}\right]$$
$$=S_0\,\frac{\ln h}{I}\sum_{i=1}^{\gamma}\frac{\alpha_i}{k+r_i+\frac{\ln h}{I}} \tag{63}$$
$$\left\langle\left[k+r_i+\frac{\ln(pg)}{I}\right]\left\{\exp\left[k+r_i+\frac{\ln(pg)}{I}\right]t\right\}-\frac{\ln\frac{(pg)}{h}}{I}\,e\exp\frac{\ln\frac{pg}{h}}{I}\,t\right\rangle$$

The assumption on which we operate here implies that the cells which undergo division at time t are those and only those which divided at time $t-I$ Therefore, we have the following relationship

$$Sm_{(t)}=\frac{1}{h}\,Sm_{(t-I)}\,e^{-kI}+\frac{h-1}{h}\,S_{(t)}$$

or

$$Sm_{(t-I)}\,e^{-kI}=hSm_{(t)}-(h-1)S_{(t)}, \tag{64}$$

and substituting in (63) we obtain

$$Sm_{(t)}=S_0\sum_{i=1}^{\gamma}\frac{\alpha_i\,e^{r_it}}{k+r_i+\frac{\ln h}{I}}\left\{\frac{\ln h}{c(1-h)I}\left[k+r_i+\frac{\ln(pg)}{I}\right.\right.$$
$$\left.\left.-\frac{\ln\frac{pg}{h}}{I}\exp-\left(k+r_i+\frac{\ln h}{I}\right)t\right]+1\right\} \tag{65}$$

Substituting $Sm_{(t)}$ into equations (38) and (40), we obtain equations which are applicable under these conditions

$$Sd_{(t)}=S_0\,\frac{g(1-p)\ln(pg)}{\rho(pg-1)I}\sum_{i=1}^{\gamma}\frac{\alpha_i\,e^{r_it}}{k+r_i+\frac{\ln h}{I}}\int_0^t\left\{\frac{\ln h}{c(1-h)I}\left[k+r_i\right.\right.$$
$$\left.\left.+\frac{\ln(pg)}{I}-\frac{\ln\frac{pg}{h}}{I}\exp-\left(k+r_i+\frac{\ln h}{I}\right)(t-x)\right]+1\right\} \tag{66}$$
$$\left\{\exp-\left[k+r_i+\frac{\ln(pg)}{I}\right]x\right\}\phi_{(x)}\,dx$$

and

$$
\begin{aligned}
S_{q(t)} = {} & \frac{S_0}{(q+\rho q')I}\sum_{i=1}^{\gamma}\frac{\alpha_i e^{r_i t}}{k+r_i+\frac{\ln h}{I}}\left\langle q\ln h \right. \\
& \cdot\left[1-\exp-\left(k+r_i+\frac{\ln h}{I}\right)t\right]+\frac{q'g(1-p)\ln(pg)}{pg-1} \\
& \int_0^t\left\{\frac{\ln h}{c(1-h)I}\left[k+r_i+\frac{\ln(pg)}{I}-\frac{\ln\frac{pg}{h}}{I}\right.\right. \\
& \left.\left.\exp-\left(k+r_i+\frac{\ln h}{I}\right)(t-x)\right]+1\right\} \\
& \left.\cdot\left\{\exp-\left[k+r_i+\frac{\ln(pg)}{I}\right]x\right\}\phi_{(x)}dx\right\rangle
\end{aligned}
\tag{67}
$$

In the steady state where $p = 1/g$, $\rho = [(g-1)L]/I$, and $c = 1/I$ (*vide supra*), we have

$$
\begin{aligned}
S_{m(t)} = S_0 \sum_{i=1}^{\gamma}\frac{\alpha_i e^{r_i t}}{k+r_i+\frac{\ln h}{I}}\left\{\frac{\ln h}{1-h}\left[k+r_i+\frac{\ln h}{I}\right.\right. \\
\left.\left.\exp-\left(k+r_i+\frac{\ln h}{I}\right)t\right]+1\right\},
\end{aligned}
\tag{68}
$$

$$
\begin{aligned}
S_{d(t)} = \frac{S_0}{L}\sum_{i=1}^{\gamma}\frac{\alpha_i e^{r_i t}}{k+r_i+\frac{\ln h}{I}}\int_0^t\left\{\frac{\ln h}{1-h}\left[k+r_i+\frac{\ln h}{I}\right.\right. \\
\left.\left.\exp-\left(k+r_i+\frac{\ln h}{I}\right)(t-x)\right]+1\right\}e^{-(k+r_i)x}\phi_{(x)}dx,
\end{aligned}
\tag{69}
$$

and

$$
\begin{aligned}
S_{q(t)} = {} & \frac{S_0}{qI+(g-1)q'L}\sum_{i=1}^{\gamma}\frac{\alpha_i e^{r_i t}}{k+r_i+\frac{\ln h}{I}}\left\{q\ln h \cdot\right. \\
& \left[1-\exp-\left(k+r_i+\frac{\ln h}{I}\right)t\right]+q'(g-1) \\
& \int_0^t\frac{\ln h}{1-h}\left[k+r_i+\frac{\ln h}{I}\right. \\
& \left.\left.\cdot\exp-\left(k+r_i+\frac{\ln h}{I}\right)(t-x)\right]+1\right\}e^{-(k+r_i)x}\phi_{(x)}dx
\end{aligned}
\tag{70}
$$

Equations (69) and (70) represent an improvement over those previously derived [reference 8, equations (25) and (36)] because no approximation has been made here in regard to the probability that DNA which is formed at time θ will be present at a later time t in the cells destined to die.

DISCUSSION

The present analysis clearly demonstrates the complexity of the problem that confronts the investigator who wishes to determine the life span, generation time, and other proliferation characteristics of cells. An inspection of the equations which have been derived shows that neither the life span nor the generation time can be obtained from determinations only of the number of cells. This is true even when the mitotic time is known and such determinations are combined with simultaneous determinations of the mitotic index, or even when colchicine is used to arrest mitoses over a known period of time. Such determinations can yield only the mean rate of cell formation and the mean rate of cell death, but can give no information on the generation time or the life span of cells. If, however, the proliferation characteristics remain constant and the mean number of cells formed per mitosis, g, is known, then the number of *cells destined to divide* can be determined from the rate of cell formation, if the mean generation time is known and vice versa. In this case the probability p that cells formed at any time will ultimately divide can be determined from equation (9) or (13). Equation (10) can then be solved (for $t \geq \tau$ when $\eta = 0$) to yield the function $\phi_{(t-\theta)}$, i.e., the probability of survival of cells formed at time θ. Equation (11) shows that in *pre-established exponential proliferation* the ratio $M_{(t)}/P_{(t)}$, though constant, depends on the distribution of the life spans of the individual cells rather than on the mean life span, in contrast to the *steady state*.

Thus, the determination of the life span of cells from determinations of their number can only be done if the generation time of the cells is known or if the *cells destined to divide* are recognizable. In addition, the number of cells produced per mitosis and the rate of cell formation are needed. The latter can be only approximated by completely arresting and counting the mitoses over a known period of time or by the use of the mitotic index if the mitotic time is known. For this reason, life spans obtained by this method should be regarded only as *approximations*.

The application of the equations of the DNA specific activity in the generalized nonsteady state requires that the functions $g_{(t)}$, $p_{(t)}$, $\eta_{(t)}$, $q_{(t)}$, $q'_{(t)}$, $q'_{0(t)}$, and $u_{(t)}$ must be known and that the functions $\phi_{(t-\theta,\theta)}$ and $\pi_{(t-\theta,\theta)}$ must be independent of θ. The last condition implies that the life span and the generation time must be constant, i.e., independent of the time of the formation of the cells. Although the function $g_{(t)}$ may be obtained by observing the cells in mitosis and computing the mean number of poles per mitosis, the functions $p_{(t)}$, $q_{(t)}$, $q'_{(t)}$, $q'_{0(t)}$, and $\eta_{(t)}$ can be obtained only if the *cells destined to divide* are recognizable as such at the time of their formation. Furthermore, there is no method available to determine the function, $u_{(t)}$, of reutilization of intact DNA from dead cells. However, even if these functions were obtainable, substitution into the equations of the generalized nonsteady state would yield equations that would be difficult to manage. Thus, *the analysis of the generalized, nonsteady state is impractical,* due to technical and mathematical difficulties. It is, therefore, highly desirable to study the cells when their proliferation characteristics remain constant, i.e., when the number of cells either changes exponentially or remains constant. Thus, in cultures in vitro, the isotope should not be added to the medium before such constant characteristics are established. Similarly for in vivo studies one should select cases in which there is evidence that these conditions are met.

It should be emphasized here that knowledge of the specific activity, $Sm_{(t)}$, of the DNA of cells in mitosis is necessary for the application of these equations. In the case where $Sq_{(t)}$ and $Sd_{(t)}$ or $Sr_{(t)}$ can be obtained experimentally, knowledge of $Sm_{(t)}$ is sufficient for the determination of $\pi_{(t)}$ and $\phi_{(t)}$. In this case it is immaterial whether reutilization of DNA occurs or not, and knowledge of the specific activity of the DNA precursor, $S_{(t)}$ is not required if $Sm_{(t)}$ is determined experimentally with the aid of equation (59). Furthermore, since the constant μ cancels out when $Sq_{(t)}$, $Sm_{(t)}$, and $S2_{(t)}$ are taken from equations (57), (58), and (60), direct determination of the specific activity of the DNA is not needed, and the grain counts from radioautographs are sufficient for the application of the equations (provided that all the radioautographs have been identically processed). An exception occurs in the case where each cell has a generation time practically identical and constant to that of every other cell and the reutilization of intact DNA from dead cells is negligible. In this case equations (66) to (70) may be applied if $S_{(t)}$ is known.

Tritiated thymidine appears to be the best compound yet available for the determination of $Sm_{(t)}$, since it is incorporated only into the DNA and since the low beta energy of tritium permits high resolution in radioautographs. It is, therefore, the substance of choice for in vitro cultures and experimental animals. Its only limitation in these cases is that it should not be used at concentrations which may cause radiation damage during the experimental period. One should be cautious, however, in using this substance in human beings because of the relatively greater radiation risk.[1] In this respect, stable isotopes are ideal for use in human beings, but no information on $Sm_{(t)}$ can be obtained with their use.

It should be emphasized that the equations which have been derived here are basic equations. The application and the interpretation of the results of these equations can not be made indiscriminately, but they require a thorough understanding of the dynamic behavior and of the homogeneity or heterogeneity of the cell population or the tissue under study. In the case of the hemopoietic tissue, for example, one is confronted with a very complex tissue consisting of several distinct cell series, such as the granulocytic, lymphocytic, monocytic, etc. Some of these cells proliferate predominantly in the bone marrow, others in the lymph nodes, thymus, etc. The *cells destined to die* may spend some time at the sites of their formation, eventually enter the blood stream, and migrate to and from the tissues, death may occur anywhere in their journey. It is thus apparent that it is not possible to obtain a sample which represents the cells of all ages in true total body proportions from blood, marrow, or elsewhere. For this reason, the interpretation of results obtained by sampling the leukocytes of the blood is problematic. Ordinarily, practically all the blood leukocytes may be assumed to be destined to die. Thus, $Sm_{(t)}$ which is needed for the application of the appropriate equation (38 *or* 51), can be obtained only by sampling the cells found in the blood forming organs and applying equation (59) Furthermore, the function $\phi_{(t)}$, which can then be obtained from equation (38 or 51), should be interpreted as the probability that cells formed at zero time will be present in the blood stream at time t In other words, it is the product of two factors the probability that cells which were formed at zero time would have entered the blood by time t and the probability that they will still be present in the blood stream at that time The probability, $\phi_{(t)}$, that cells formed at zero time will still be present in the blood forming organs at time t (from which the mean time spent in the blood forming organs can be obtained) may be determined by sampling the blood forming organs and solving the system of equations (36), (42), (43), and (44) or (49), (54), (55), and (56), if no reutilization of intact DNA from dead cells occurs However, if reutilization of such DNA does occur, then u must be known, and equations (34) and (35) or (47) and (48) should replace equation (36) or (49) in the above system. Assuming further that no cell death occurs in these organs, $1 - \phi_{(t)}$ is the probability that they have entered the blood stream Thus, dividing the probability that these cells will be present in the blood stream at time t [determined by applying equation (38) or (51) to data obtained from the blood leukocytes] by this last probability, and subtracting the quotient from 1, we obtain the probability that cells formed at zero time disappear from the blood stream by time t This probability, in turn, is the result of the probability that the cells will die prior to t and of the probability that they will have left the blood stream and migrated into the tissues It, therefore, gives only the fraction of the leukocyte life span which is spent in the blood stream If, however, there is evidence indicating that the mixing time for blood and tissue leukocytes is quite short in comparison to their life span, then blood and tissues may be considered a single pool, and the life in the blood stream may be regarded as the true leukocyte life span If in addition, the mixing of the leukocytes of the whole body is very rapid, as it may possibly be in some cases of leukemia, then sampling the blood alone should be sufficient to study the proliferation characteristics of the leukocytes It should be noted that if leukocytes, regardless of age, enter or leave the blood stream in a sudden and unpredictable way, as may occur under the influence of epinephrine or histamine, then the above discussed probabilities are functions not only of the age of the leukocytes but also of the time of observation Under these conditions the equations cannot be solved, and the problem defies analysis

The above discussion shows the extreme complexity of the problem of determining the proliferation characteristics of leukocytes in vivo, compared, for example, with cultured cells, where the entire population can be sampled In addition to the mathematical difficulties, there are also technical difficulties, such as (a) the necessity for frequent sampling of the blood forming organs, which is impractical, (b) the difficulties involved in securing such samples free of blood, or in correcting for its presence, and (c) the need of radioautographs and of accurate grain counts of individual cell nuclei for the determination of $S_{m(t)}$ and of the specific activity of cells under study, which is not a simple task.

An approximate method bypassing the need for sampling the blood forming organs can be followed if a steady state is maintained and if the following assumptions can be made (a) the generation time of every leukocyte of the series under study is practically the same (b) reutilization of intact DNA from dead cells does not occur or it is negligible, (c) the specific activity $S_{(t)}$ of the DNA precursor is closely approximated by that of a blood constituent which can be isolated and studied and (d) the cells that enter the blood stream remain there long enough to obtain a plateau in the curve of the specific activity of the DNA $Sd_{(t)}$ In this respect, it should be noted that a true plateau will never actually be reached even when the isotope is available to the cells for a very short period of time, because labeled DNA is transferred from the dividing cells to the *cells destined to die* in an exponential manner A plateau may be approximated however if the time spent in the blood stream is many times greater than the generation time of the leukocytes and the data are corrected for the physical decay of the isotope If such is the case equation (68) may be used to determine $Sm_{(t)}$ Then the ascending section of the curve $Sd_{(t)}$ (i e the section prior to the plateau) will be given by *an equation similar to* (51) *in which* $\phi_{(x)}$ *represents* the probability that the leukocytes enter the blood stream prior to an age x The remainder of the analysis may then proceed as the foregoing one

In concluding the main points that have been brought out by this analysis are that (a) knowledge of the rate of isotope incorporation into the DNA of cells makes it possible to determine the life span and generation time of cells which are either in a steady state or show an exponential change in number provided that g, q q and u are known (b) the complexity of the equations of the generalized nonsteady state make this method as well as any other indirect method that may be used for this purpose quite impractical and (c) the dynamic behavior and the homogeneity or heterogeneity of the tissue under study must be known so that appropriate use of the equations can be made

SUMMARY

The dynamics of cell proliferation in the nonsteady and steady state have been theoretically analyzed It is shown that when the proliferation characteristics remain constant the number of cells changes exponentially, and the ratio of the *cells destined to divide* to those *destined to die* remains constant It is concluded that mere observation of an exponential increase of the number of cells is insufficient evidence for excluding cell death The theoretical analysis of the kinetics of isotope incorporation into the DNA indicates that the method is suitable for the determination of the distribution and the mean of the life span and of the generation time of cells that are either in a steady state or show an exponential change in number The complexity however of the equations of the generalized nonsteady state makes the use of this method as well as any other indirect method quite impractical The use of the equations, particularly in reference to their application in the study of the hemopoietic system as well as the limitations of the method have been discussed

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The Description of Steady-State Kinetics

By Henry Quastler

IN THE course of this meeting it has become painfully obvious that communication concerning cell population kinetics is difficult. The difficulties might be somewhat relieved by adherence to a strict scheme of analysis. Such a scheme is proposed here. It refers only to the simplest case: steady state conditions in a population of large size. (If transients are to be considered, then one has to introduce some age functions, such as those discussed by von Foerster, page 382, or Maurer, page 231, and if the population or some important subpopulation is of small size, then statistical refinements such as those discussed by Harris, page 368, will be indicated.)

If we are concerned only with a single category of cells, then we shall wish to know the following four kinetics parameters: (i) a size parameter, n, the number or mass, absolute or relative, of cells of the kind considered; (ii) a time parameter, t, the mean time of sojourn, or mean time elapsing between a cell acquiring and subsequently losing the characteristics of the category under consideration; (iii) two flow parameters, λ_{in} and λ_{out}, denoting number or mass (absolute or relative) of cells per unit time entering or leaving the category considered, where entrance can be by birth or changing over from another category, and exit by death or changing into another category.

The steady state condition imposes the following two constraints:

$$\lambda_{in} = \lambda_{out} \tag{1}$$

$$t = n/\lambda_{out} \tag{2}$$

Therefore, one has to measure only two parameters and can then compute the two others. There are five different but equivalent ways of measuring two parameters which will suffice to determine the whole system: one can measure n and t, or n and λ_{in}, or n and λ_{out}, or t and λ_{in}, or t and λ_{out}.

The situation gets rapidly more complicated as the number of compartments considered increases. For a two-compartment system, we need 10 parameters for a complete specification:

2 size parameters, n_1 and n_2,
2 time parameters, t_1 and t_2,
a total of 6 flow parameters, λ_{ij}, where the first subscript refers to the "source" and the second to the "sink" of the flow; thus, the two birth parameters λ_{b1} and λ_{b2} designate the number of cells of type 1 and 2 produced in a unit of time, the 2 death parameters λ_{1d} and λ_{2d} the number of cells of either kind dying in a unit of time, and the 2 transition parameters λ_{12} and λ_{21}, the rate of change from type 1 to 2, and from type 2 to 1, respectively.

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The 2 steady state conditions for each compartment

(1a) $$\lambda_{\text{in}} - \sum_j \lambda_j = \lambda_{\text{out}} - \sum_j \lambda$$

(where i can stand for 1 or 2 and j for 1 2 b or d)

(2a) $$t_i = n / \sum_j \lambda$$

yield a total of 4 equations Hence one has to measure 6 parameters and can then compute the remaining 4 There are 12 different ways of picking a set of 4 parameters sufficient to specify the first compartment and 5 ways of selecting an additional 2 parameters to specify the second compartment

In general if a system has R compartments then there will be R each of size time birth and death parameters and $R(R-1)$ transition parameters for a total of $R^2 + 3R$ If steady state conditions hold then there will be $2R$ equations which reduce the number of independent parameters to $R^2 + R$ There are $R(R+4)$ different ways of selecting a sufficient set of parameters for measurement to specify the first compartment $(R-1) \times (R+3)$ different sets for the second etc If sizes are to be measured in fractions of the whole system then another constraint is introduced

$$\sum n = 1$$

which reduces the number of parameters to be directly measured by 1 and multiplies the number of effective ways of doing so by R For instance in our analysis of the kinetics of the intestinal epithelium we used 5 compartments (synthesis phase premitotic plus mitotic phase postmitotic phase transition phase villus phase) which calls for 40 parameters There are 10 steady state conditions and an eleventh constraint since compartment sizes are given as fractional counts Therefore 29 parameters had to be estimated some on the basis of general—or previous—knowledge some on the basis of specific experiments Since it is difficult to keep track of an operation of this complexity it is helpful to tabulate all parameters involved make a numbered list of all estimation procedures used (indicating whether they are based on general information special information or computation) and set the appropriate number next to each parameter in the table We use the following scheme

$i = 1\ 2$ R where 1 — (name of first compartment)
2 — (name of second compartment)

•

R — (name of Rth compartment)

Size	Time	Flow Parameters (rows inflow columns outflow)				
n_1	t_1	—	λ_{21}	λ_{31}	λ_{R1}	λ_{b1}
n_2	t_2	λ_{12}	—	λ_{32}	λ_{R2}	λ_{b2}
n_R	t_R	λ_{1R}	λ_{2R}		—	λ_{bR}
		λ_{1d}	λ_{2d}		λ_{Rd}	—

sources and sinks of matter and energy found in closed systems there may be an exchange with the outside. When this exchange with the outside becomes steady the internal sources and sinks must eventually disappear and the currents of matter and energy passing into any internal locale will then just balance the outflow. In such a steady state system there may still be currents, so that each reaction is not necessarily balanced by its inverse as in the equilibrium state but none-the-less the entropy has been maximized as far as the imposed interaction with the outside allows and this is accomplished by eliminating all internal sources and sinks. This principle of the maximizing of the entropy of open systems in a steady state by the disappearances of internal sources and sinks constitutes, together with Onsager's relations between current and driving force, the contribution which irreversible thermodynamics can make to our understanding of population problems. Onsager's relations are a consequence of the principle of detailed balance. The theory of absolute rates of elementary reactions together with chain reaction theory is the general theory of nonequilibrium systems which includes irreversible thermodynamics as the limiting case for these systems which are only slightly displaced from equilibrium.

The problem of determining total cell populations of the type found in the blood is much like taking the census in a city. Individuals are being born, are dying and are changing their appearance along with their occupations so that counting them by measuring travel on the subway is full of pitfalls. Even if you radioactively mark them at intervals and watch the rate at which marked individuals disappear you *aren't sure whether they died or just quit* riding the subway. Taking the census of residence districts and watching the movements of marked individuals helps round out a complex picture.

Only as we build hypotheses tying the facts together can we hope to organize an otherwise jumbled picture. Since a theory involving molecules must require these molecules to exhibit reasonable behavior, it follows that much of chemistry is required background. Leukemia, like cancer in general, apparently involves a damaging of the templates which are concerned with manufacturing growth inhibitors or with changes increasing the production of growth promoting substance. In either case there is an upset in the delicate balance in the direction of uncontrolled growth. These defective templates are passed on from mother to daughter cells. Osgood and others have assembled many of the facts he has used in elaborating his views along these lines.[2, 3] Since normal cells in man double their number only about five times from birth to maturity, any marked decrease in the inhibition of growth of any undifferentiated cell or an increase in the growth factor brings disaster. Rashevsky's mathematical formulation of a model[4] has the merit of leading to a definite formalism and being couched in terms of a growth factor characteristic of each cell whose growth is in turn inhibited by a factor elaborated by all undifferentiated cells. As undifferentiated cells decrease in number with age fast growing cells may cause trouble by escaping from control of an inhibitor elaborated by undifferentiated cells. Mutation of a cell giving it a larger growth factor would, according to this view, send it out of control at an earlier age, i.e., whenever the decreasing amount of inhibitor can no longer hold rapidly dividing cells in check. Some carcinogens by destroying the inhibitor supplied by neighboring cells may cause uncontrolled growth in a fast growing cell, while others may promote growth directly or by mutation into a fast growing cell.

Actually, further experimental research is necessary to distinguish between the various points of view outlined and other possible models. Fortunately, therapy doesn't require the knowledge needed to undo the changes of disease. It may simply *direct these changes along a different course*. Surgery removes the tumor while x rays and certain drugs help by interfering with cell division. Prevention, however, requires a still deeper understanding.

H. R. Warner: We are using an analog computer to explore various mathematical models of leukocyte kinetics. We treat the total granulocyte population as a compartmentalized system. The first compartment (M_1) is composed of cells capable of undergoing mitosis. Obviously this compartment is composed of several cell types. Only uni-formity of initial labeling and of fractional turnover rates of these cell types would permit treatment of this whole population as a single compartment the specific activity of which would decay exponentially from the time of labeling The next major compartment (M_2) is composed of cells *which are maturing that is cells which* are no longer capable of dividing and yet are not freely exchanged with the mature cells in blood This compartment may be thought of as a tube through which cells move and leave in the same sequence as they entered A third marrow compartment (M_3) may exist This is a population of mature cells which enter the blood randomly That leukocytes are removed randomly from blood is evident from the data on the kinetics of in vitro labeled leukocytes presented by Maurer [6] The equations for the model just described are

$$\text{(1)} \qquad \frac{dCm_1}{dt} = \frac{K}{Vm_1} Cm_1$$

$$\text{(2)} \qquad Cm_{2(t)} = Cm_{1(t-t_1)}$$

$$\text{(3)} \qquad \frac{dCm_3}{dt} = \frac{K}{Vm_3} (Cm_2 - Cm_3)$$

$$\text{(4)} \qquad \frac{dC_B}{dt} = \frac{K}{V_B} (Cm_3 - C_B)$$

where *C is concentration of label in various* compartments, V is total cells in a compartment t_1 is time for maturation K is rate of cell production and M_1 M_2 and M_3 refer to the 3 marrow compartments and *B* the blood compartment

In the paper given by Maurer[6] it was shown that this model was able to accurately predict the time-course for the specific activity of leukocytes in blood following the administration of P^{32} labeled DFP The model could be made to fit the experimental data by assuming that initially the cells in the *marrow* were labeled uniformly at a level of about 30 per cent of the initial specific activity of leukocytes in blood This assumption although not validated by direct observation would seem reasonable, since leukocytes in blood *are exposed to the highest concentration* of the label at the time it is administered intravenously Of particular interest is the fact that the parameters used *to* fit the data with these equations are in agreement with values for the same parameters obtained by other workers using an independent approach [4]

Although this model satisfactorily predicts the time-course of the DFP^{32} label in blood leukocytes the question as to whether these equations describe white *blood cell kinetics must still remain open* For this reason the ability of the model to predict the time-course of label in blood leukocytes following the administration of H^3 labeled thymidine[7] or P^{32}-labeled inorganic phosphorus[8, 9] was tested A curve closely fitting the experimental data was *readily obtained from* simultaneous solution of the model equations by simply assuming the M_1 compartment to be the only one initially labeled

A fundamental discrepancy however appears to exist between the parameters employed to predict these data and the parameters used in fitting the DFP^{32} curve This difference lies in the apparent duration of the maturation process This process appeared to occupy ten days in the case of the DFP^{32} experiments whereas with the other labels maturation occupied only three to five days Whether this discrepancy represents inadequacy of the model or a difference in the experimental conditions cannot be decided on the basis of the facts currently available

The material just presented represents a single example of the role mathematics may be expected to play in advancing a field such as the one under *discussion* This role consists of providing the means for expressing a hypothesis in such a way as to allow quantitative testing *of its ability* to explain observed data The analog computer in our hands has served as a valuable tool to provide rapid solutions in graphical *form for ready comparison* with experimental curves

P Meier The previous speakers have given you all *too brief* a view of certain elements of population theory but obviously in the time allotted it would be inappropriate *for me to try* to discuss further specific mathematical techniques Instead, I should like to comment on the role of *mathematics and mathematical models* in biologic investigations.

Mr Cornfield emphasized the importance of mathematical models explicit or otherwise, in providing a framework within sources and sinks of matter and energy found in closed systems there may be an exchange with the outside When this exchange with the outside becomes steady the internal sources and sinks must eventually disappear and the currents of matter and energy passing into any internal locale will then just balance the outflow In such a steady state system there may still be currents so that each reaction is not necessarily balanced by its inverse as in the equilibrium state but none-the-less the entropy has been maximized as far as the imposed interaction with the outside allows and this is accomplished by eliminating all internal sources and sinks This principle of the maximizing of the entropy of open systems in a steady state by the disappearances of internal sources and sinks constitutes together with Onsager's relations between current and driving force the contribution which irreversible thermodynamics can make to our understanding of population problems Onsager's relations are a consequence of the principle of detailed balance The theory of absolute rates of elementary reactions together with chain reaction theory is the general theory of nonequilibrium systems which includes irreversible thermodynamics as the limiting case for these systems which are only slightly displaced from equilibrium

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First, if the experimental results agree with previous predictions based on the model, we may tend to feel confident that we really understand the situation

Second, and perhaps less often recognized, is the fact that it is frequently not at all obvious what the consequences of even rather simple assumptions will be in complex systems The biologic assumptions used in the models which Dr Harris has spoken about are of the very simplest kind imaginable, yet the analysis of these models leads to unfamiliar mathematics and to quantitative results which, although quite plausible, are perhaps not immediately obvious It seems to me that without this specific model (or specific models of this kind) for analysis it may well be impossible to decide whether experimental observations are only what should be expected or whether revision of basic concepts is called for

Now, with these remarks as background, I would like to comment on two attitudes toward mathematics which I think are common among biologists and which are almost equally unfortunate

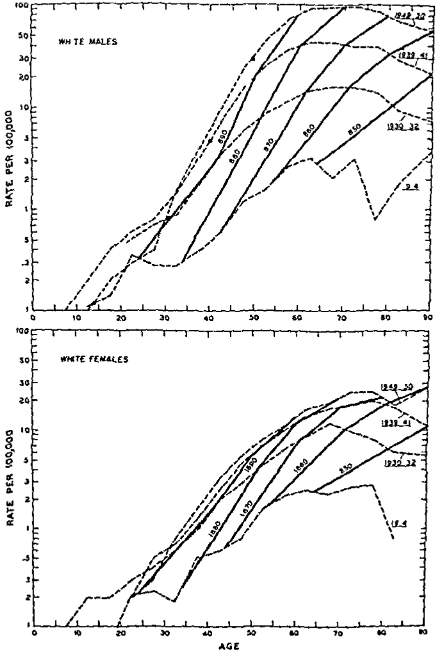
First is the negative attitude which expresses itself in the general point of view that all important findings are basically quite simple, and that if you need a lot of fancy mathematics to back up your interpretations you must be on pretty shaky ground It is doubtless true, of course, that complex mathematics can be used to cover up the data rather than to clarify them On the other hand, we have just seen how simple assumptions may lead to results that are not intuitively obvious, and if phenomena of this type are to be seriously investigated I see no escape from the corresponding mathematics

At the opposite pole there is the enthusiastic attitude which looks upon mathematics as a kind of magic which can somehow overcome the intransigence of the experimental situation and lead, practically of its own accord, to new and useful information The most common expression of this approach is in the development of mathematical models with so much flexibility and so many free parameters that almost any experimental outcome fits A typical example of this sort is the fitting of any convex dye dilution curve with a linear combination of exponential functions, and the subsequent interpretation of these exponential components as evidence for the existence of mixing pools of various sizes I don't mean to say that no dye dilution curve should ever be looked at in this way, but the mere fact that a curve can be approximated by a series of exponentials does not of itself prove the existence of mixing pools

Finally it seems to me that when scientific research in a given area becomes concerned with quantitative relationships the scientist himself has to become something of a mathematician That is, he has to know enough to clearly understand the models which represent these relationships, and understand them well enough in fact, not only to manipulate them but also to get rid of any exaggerated respect for them, and to recognize that although mathematics is one of the indispensable tools of the trade, it is a tool and not a substance

M A SCHNEIDERMAN I would like to give an example from some real data of the type of model that Dr Von Foerster was talking about I also want to comment on what Dr Meier has just said about sums of exponentials

In Discussion figure 1 age is plotted along the x axis and rate on the y axis The curve is the death rate in this case from lung cancer In this particular situation we have a set of data which relates both to Dr Von Foerster's and to Dr Harris remarks This is a situation in which the basic population in some way changes with time There was somehow a change in the extrinsic causes of death which were much more than the random element that Dr Harris put in It has been noticed that in the deaths reported from lung cancer over different years there were curves such as those in the figure, reaching a peak (at say 65–70) and then declining with increasing age This was observed in 1914, 1930–1932, 1939–1941 and 1949–1951, and involved taking a *cross section of the population* In the figure, these cross sections are shown by dashed lines This was the second form population that Dr Von Foerster dealt with, where he had a cross section of



Disc Fig 1—The data are for white males in the United States The figure is from Morbidity From Cancer in the United States, Public Health Monograph No 56 (published by U S Department of Health, Education, and Welfare, Public Health Service), by Harold Dorn and Sidney Cutler I am indebted to them for permission to use it

the population that has some erratic age distribution Looking at this cross section of the population, this graph gives the same kind of thing These data, then, seem to imply that there is an increasing risk of dying from lung cancer until one reaches a certain age, and then somehow or other the risk is reduced At that point you might die from a heart attack or some other cause instead of from lung cancer

Let us now look at the population *not as a cross section but as a cohort population,* all born in a given year and followed across time If we take the persons born in a given year and their death rates at a given age, these rates all continue to go up These are the solid lines on this figure, and they never turn down Here there is an increasing risk with increasing age for all ages—and the risk is changing (upward) with time The cohort born in 1850 had a death rate of 2 to 3 per 100,000 at age 60 to 65 The 1860 cohort had a rate of 5 to 6 per 100,000 The 1870 cohort had a rate near 20 The 1880 cohort, at age 60 to 65, had a rate near 60! There is some powerful *X* factor of Dr Harris' in operation here, but if we looked at the data in cross section only, we would certainly not see what we have seen when we looked at the cohorts

In the hematologic problem, taking out an aliquot of red cells, labeling with Cr^{51}, putting them back in and following them, we have the cross section population Trying to get initial labeling of cells (or their DNA) with tritium-labeled thymidine is almost like following a cohort population These different approaches lead to different conclusions You have to be very careful to define the population with which you are working, and to deal with this population in the proper manner Only when Dr Harris' X is missing are these populations interchangeable

The second order remark on Dr Meier's comment is that I agree with him wholeheartedly but for one thing You can always take one of these curves and show decay with curvature, and show it not as the sum of one or two or three or four exponentials, but as the sum of an infinite number of exponentials This implies a different (and more meaningful?) biologic model, and the mathematics, surprisingly, are much easier

J S ROBERTSON Much of the work that has been discussed has involved the use of tracers A point which I would like to emphasize is that in using tracers we are not usually concerned with the behavior of the labeled material per se The important question is, what can the tracer or the labeled material show us about what goes on in the unlabeled counterpart? In steady-state situations it is not apparent that anything is happening to the unlabeled material, but introduction of a tracer may show that material is being transferred from one compartment to another This is where the use of a model enters the picture and where models are important The role of the model is to give a basis for establishing the rational connection between the behavior of the tracer and the behavior of the unlabeled material If the mathematical equations describing the model are established on a rational basis, the curves describing the behavior of the labeled material can be fitted to solutions and thus the parameters describing the behavior of the unlabeled counterparts can be established in terms of the model Dr Von Foerster has emphasized that mathematical manipulations of the data, correctly used, only rearrange the information without adding to it A model provides the framework by which the description of the tracer's behavior can be translated into that of the unlabeled material

As Dr Quastler pointed out, after you get more than a few compartments in the system, it is impossible to solve the equations in sufficiently explicit terms to be of use in interpreting the data Explicit solutions are unnecessary, however, because the electrical analog computers are capable of establishing the necessary relationships Another role of the model, then, is to provide a way of programming electrical computers I expect these machines to assume increasingly important roles in the interpretation of data on the behavior of tracers in hematologic studies

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Conference Summary

By Eugene P Cronkite M D

IT IS impossible to summarize this conference adequately and to do justice to all of the participants and the wide range of topics covered Of necessity certain points which appear pertinent to me will be dwelled upon at the risk of neglecting equally important facets

First the various chairmen performed admirably in keeping their programs to the subject and on schedule so that there was ample time for free discussion from the floor I believe the Hematology Study Section and its Organizing Committee can be justifiably proud of the fact that open discussion was animated and there was not a single lull in the course of the three days At the closing of each session there were still many questions to be asked The informal discussions continued animatedly far into each night

In specific comment I will take the prerogative of first criticizing my own opening comments The scheme proposed was obviously a gross oversimplification It was erected to be destroyed and in that respect was not a complete failure In retrospect it is quite obvious that there were many hidden assumptions of which we were not properly aware The calculations for sizes of the various parts of the neutrophil compartments were presented too briefly and without sufficient description This resulted in misunderstanding Bond s value for an *upper limit* for time in the peripheral blood was used This value when used in conjunction with the estimate from the mitotic index for generation time of the last myelocyte to divide yields an estimate of the number of myelocytes in this category This value is a *minimum* It can scarcely be smaller This number tells nothing about the average size or the maximum possible The confusion that resulted demonstrates the necessity for person to person discussion and serves to indicate the intricate nature of problems surrounding the establishment of the duration and size of each phase in the life cycle of the neutrophil

Dr Andreasen of Denmark pointed out that the mitotic index determined in tissues is unreliable He presented a method for the reliable determination of the mitotic index in hemopoietic tissues by the use of suspensions of cell nuclei He commented upon the presence of phagocytosis of nuclear material in areas of lymphocytic proliferation and believes that significant numbers of lymphocytes go to the epidermis and gastrointestinal epithelium I believe he postulated that the lymphocyte may transport DNA to areas of cell division If true this assumption could be confirmed by labeling technics suitable for autoradiography There is no doubt that Andreasen s observations point out the difficulties in describing the behavior of the lymphocyte in kinetic terms

Dr Leblond of Canada demonstrated the limitations and usefulness of the mitotic index and illustrated how the technic in conjunction with cell counts

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From the Medical Research Center Brookhaven National Laboratory Upton N Y can be used to establish a model for small lymphocyte production from the reticular cell to the final product by variable repetitive divisions and maturation. His studies are based on fixed tissue sections and it appears that study of imprints with Romanowsky staining would also complement his studies.

The lymphocyte dilemma of the British seems more apparent than real. The data of Doctors Yoffey and Gowans are excellent. I observe their technics and quantitative expression of data with admiration. I believe that in the long run their data will prove to be compatible. The differences in interpretation may in part be due to species differences but primarily I believe they derive from differences in analysis of the data. At any rate these differences cannot be resolved by polemics. I suspect that critical experiments or newer analytical approaches arising from this conference will resolve such difference of opinion. There seems to be little doubt about recirculation in part of the small lymphocyte. The magnitude to Gowans is large, to Yoffey small. Neither has resolved the problem posed by Hamilton some years ago about possible reutilization of DNA. Andreassen also apparently subscribes to this idea in part. I do not see how one can avoid concluding that some lymphocytes are long lived as Ottesen and Hamilton published earlier.

The combined presentations of our Scandinavian colleagues Andreasen and Fichtelius emphasize the danger of oversimplification in describing the behavior of the lymphocyte. The lymphocyte life cycle and function is a complicated affair, to be sure. Quite obviously no one simple explanation is adequate to explain the behavior of autologous, homologous or heterologous transfused lymphocytes or, for that matter, their behavior in vivo after labeling by isotopic procedures suitable for autoradiography. Possibly in situ death near areas of proliferation is as prominent for lymphocytes as it is proposed by Stohlman for erythrocyte precursors. Phagocytized nuclear labeled material has been observed by Brecher early after labeling, suggesting the death of cells soon after DNA synthesis. Andreasen's and Kelly's data seem consistent with an in situ death of a portion of the proliferating cells of lymphocytic tissue.

Kelly, from the extensive studies of her group, believes that the distribution of cells in thoracic duct lymph is the same as in the lymph nodes. This would be most helpful if true but is open to question. She and her associates feel, as does Leblond, that the large lymphocyte produces the medium, which in turn produces the small variety. However, this has not been conclusively shown to be the case, and each could still have separate precursors. Direct observation is needed to prove the point.

In concluding a summary of the lymphocyte one must emphasize that this term represents a family of cells with different life spans and perhaps different functions but a common organ of origin. The genealogy, life expectancy and function of this family are inherently tough nuts to crack. Morphologic or functional criteria, in addition to size, are badly needed.

In respect to the granulocyte's behavior and its total life cycle more areas of agreement have appeared. The existence of an extravascular pool of neutrophilic granulocytes appears less appealing than a few years ago, or at least it has shrunk in size. Recycling of neutrophils from extravascular sites to the circulat ing blood though not eliminated from current thinking has fallen from favor The data of Craddock et al from U C L A prove the existence of a very large pool of readily available neutrophils The size of this pool is reasonably well established and is distributed primarily between an intramedullary storage site and areas of margination along capillary walls of various tissues of which the lungs may be the most important Again an extravascular pool is not eliminated but its existence and size are in question

An *upper limit* for the average stay of the neutrophil in the peripheral blood of 2 3 days has been firmly established by Bond et al The actual stay may well be substantially less In fact the data of the Utah group (Mauer Athens Cartwright and Wintrobe) Patt Craddock et al and O good all seem to converge on a rather short average stay in the blood only a few hours It is difficult for me to accept Van Dyke s very short estimate based on equilibrium values between normal and irradiated parabionts

Whether or not the Brecher and Stohlman postulate of *in situ* death of some cells during proliferation applies to granulocytopoiesis is unclear as is the ratio of the size of the intramedullary pool of granulocytes to the freely circulating mass of granulocytes However a large readily available pool of granulocytes is evident It appears that the use of H^3 thymidine and kinetic analyses based on autoradiographic observations will finally answer these problems providing the interpretation is not confounded by uncertainties in the morphologic identification of cells in the marrow Identification of cells in autoradiographic preparations is not satisfactory and this is an area where technical development is needed

Patt s studies on H^3 thymidine labeling in hemopoietic tissues of the dog introduce another species and show further useful methods of analysis Bond and Patt differed in the method of interpretation of the appearance of labeled neutrophils in the blood Patt s approach of observing the disappearance of specific labeled cell populations appears to offer a better method of estimating the actual average time in the blood The differences in percentage labeling of marrow and peripheral blood of man and the dog demand an explanation The difference may be technical or the species may just behave differently in re pect to hemopoiesis These are important problems to resolve since many studies can be performed on the dog that are rarely possible to perform on patients

Mauer et al of Professor Wintrobe s group have taken advantage of the service of an analog computer to build and test mathematical models for leukokinetics based on their DFP^{32} labeling studies Certain observations are clear-cut Their data clearly have three phases The first phase is be t interpreted as a rapid random clearance of neutrophils from the blood and appears consistent with Patt s observations and Brecher s published interpretation Their second phase having to do with maturation has a duration that is too long if one accepts the H^3 thymidine data of Bond and Patt as being reliable indicators of maturation transit time Their third phase is believed to e timate myelocytic generation time and is consistent with computations from mitotic index but not consistent with Patt s thymidine data These discrepancies can be resolved only by further experiments The kinetic model and testing technic will become more useful as data accumulate and may ultimately be the only way to answer the problems that are not subject to direct experimentation

Kline critically reviewed neutrophil life span technics, pointing out that certain of them faithfully give short life spans, others intermediate life spans, and still others long life spans Kline s own data when analyzed by use of the Volterra equations give an average life span of 13 days Whereas there is no doubt about the validity of the data, there is a serious question as to whether this analysis is appropriate Both Hamilton and Cartwright have pointed out that use of the Volterra equations demands a finite life span, and that if there is a random loss this approach does not apply

Brecher rather conclusively demonstrated that atabrine is useless as a label of leukocytes since the fluorescent dye exchanges rapidly and is primarily a cytoplasmic and not a nuclear label It appears safe to disregard the published data on life span recycling of cells etc that are based on fluorescent dye technics This is a positive accomplishment since the numbers based on fluorescent dye technics have been used extensively in computations and as evidence for recycling of cells Whether all fluorescent dyes are as unreliable as atabrine is not clear Diaminoacridine is a good nuclear dye

Tritiated thymidine, a specific DNA label suitable for autoradiography when incorporated at time of DNA synthesis prior to cell division did not have its debut at this conference Its value in studying chromosome replication was shown soon after its synthesis by Dr Hughes about three years ago at the Medical Research Center, Brookhaven National Laboratory Its value in the study of human and animal cell proliferation has been exploited by the Brookhaven group and its use has spread widely This conference has shown clearly that tritiated thymidine is not a panacea for all the difficulties in the study of cell proliferation Its areas of usefulness and limitations have become quite apparent Questions of possible perturbation of cell proliferation by tritiated thymidine and the dose levels where such perturbations may be produced are not yet resolved Further research is needed in this vital area Methods of interpretation of H^3 thymidine labeling data of DNA and cells are emerging and appear consistent between laboratories

Many questions were asked from the floor about the stability and exchangeability of tritium labeled thymine in DNA Hughes on the chemical side and Leblond, on the cellular side certainly convinced me that DNA is immortal in mature cells and that the thymine does not exchange from DNA nor the tritium from the thymine to any detectable degree after DNA synthesis These appear to be two solid irrefutable facts that make one feel more secure in the use of tritiated thymidine for the study of cell proliferation However on the pessimistic side, the question of possible radiation injury of precursor cells by H^3 thymidine in the amounts used was neither shown nor disproved This is a subject that deserves urgent attention by all who propose more extensive use of H^3 thymidine, not only because of the possible late hazard (carcinogenesis) but because of the possible perturbing effect on cell proliferation itself In my opinion the effect is negligible, but here facts are essential

The opening discussion by Hughes on DNA synthesis and degradation clearly presented the status of knowledge The N^{15} labeling of DNA in bacteria followed by ultracentrifugal separation of DNA in cesium chloride strongly suggests that the DNA double helix consists of complementary halves, one synthesizing the other prior to cell division These studies complement the earlier studies of Taylor et al on chromosome replication The chemical nature of the gene and the forces that hold DNA and chromosomes together are still unanswered

Friedkin's methyl shunt in the cycle of DNA synthesis was most interesting and I believe will open up many new areas for speculation and investigation It presumably offers a chemical explanation for a control mechanism for cell growth

Alternate pathways of thymidine metabolism were presented by Potter and are very important in interpreting the behavior of labeled cells Thomas has used DNA synthesis as a practical means of assessing viability of marrow cells which has a distinct bearing on therapeutic transplantation of preserved marrow Bendich discussed the tritium labeling in vitro of separated DNA by the Wiltzbach exchange reaction with tritium gas About one in 1000 hydrogens exchange Labeling appears to be greater with thymine than with other bases There are obvious radiochemical changes produced, as shown by changes in sedimentation constant and chromatographic characteristics He believes that the labeled DNA will enter HeLa cells, but has not ascertained whether this is direct or after degradation to fragments and then utilization of the fragments

Lajtha discussed the problems concerned with autoradiography in complex tissues The lack of "Poissoness" in grain counts indicates that DNA synthesis may have a variable rate which is biologically acceptable He also appears to be more concerned than most investigators about the possibility of radiation effects by H^3 thymidine which points up as mentioned earlier, the need for empiric studies on this question that really cannot be answered by calculation

Quastler has determined the variability of the segments of the cell generative cycle by an analysis of mitotic labeling in the small intestine of the mouse Similar studies would be useful in hematopoietic studies however the mixed cell populations growing at different rates complicate the study

Dr Dustin, of Belgium, clearly presented the colchicine arrest technic for estimation of the mitotic activity of tissues, and rigidly defined the limitations and application of the method in a manner that will be most useful to all of us in our future work

Progress has been made in understanding the platelet life span of man Odell and associates have used the radiosulfate labeling technics developed in animals for the study of platelet life span in patients Using the in vivo increase in 'specific activity' of separated platelets, they deduce a life span of 10 days in man, a value similar to that obtained by DFP^{32} earlier by others but longer than values obtained from transfusion of separated homologous platelets The implication is clear Separation procedures injure the platelet in some subtle manner that is not easily detected

Dr Bessis, of France, with clarity and beauty demonstrated an electron micrographic explanation for iron conservation and reutilization in erythropoiesis that was incorporated into Dr Stohlman's concepts on erythropoiesis before the conference ended It is believed that these observations will become another classic morphologic contribution to hematology and quite certainly showed that there is another factor to be considered in any discussion of iron kinetics All hemoglobin iron is not transported by transferrin of plasma

Alpen's very extensive Fe^{59} autoradiographic studies on erythropoiesis in the dog lead to the conclusion that erythropoietin acts by increasing the number of cells from a primitive pool that are entering erythropoiesis The observations are extensive and the arguments almost compelling, until Stohlman s equally extensive observations and lucid arguments are considered Further study will be needed to resolve these differences in opinion, many of which faded away in the course of fruitful discussion during this conference

I must omit any really serious discussion on erythropoiesis and its control There are still unanswered problems, but areas of agreement are larger Alpen and Stohlman seemed to resolve many differences The idea of cell death by red cell precursors as presented by Stohlman is apparently consistent with many of Alpen's observations with Fe^{59} autoradiography

The source of erythropoietin is unsettled The kidney still seems the most likely source, as one looks at the data of Jacobson and Naets but Erslev s data appear inconsistent Further work will be needed to resolve this problem

The chemistry of erythropoietin is developing rapidly Borsook and associates have established its mucoprotein nature With each development in its chemistry, problems of stability the nature of the protein, what determines its biological activity, and why proteins chemically indistinguishable have no activity continue to crop up More effective assay methods appear needed

Although I haven t singled out any of Dr Osgood s concepts and data for specific comment, I think it only appropriate to recognize the fact that he has been working actively in this field for the last twenty years and for many years was almost alone Today it must be gratifying to him to see the international and multidisciplinary attack on these problems and, as new technics become available, their slow resolution by experiment

It is also quite evident that hematology is benefiting enormously by the participation of people in hematologic research who a short time ago scarcely were familiar with morphologic hematology At the same time I would like to try and set the record straight in certain respects I m not much of a hematologic historian but during these three days I ve heard mention of ideas observations and schemes proposed that bore more than a superficial resemblance to some rather old concepts of Metchnikoff, Naegeli, Ferrata, Pappenheim Ranvier, Aschoff, Downey and a host of other early hematologists who had only morphology and microscopes Admittedly they resorted to polemics and their problems were not resolved Mention of their names here was sadly lacking The original literature may be inaccessible and in languages with which we are not facile, but fortunately there are available such superb books as Downey's Handbook of Hematology and texts by Bessis, Rohr and Wintrobe It appears that a closer study of these classics by the new entrants into hematologic research, and by those of longer sojourn, might be useful in getting a better perspective of where we stand and where we are going

Now that newer technics of study are available, one must ask whether we are closer to resolving our problems or whether we are further apart because of an inability to communicate to each other the significance of the transformation of our observed facts (immortal) into models (ephemeral) to explain the whole process of hematopoiesis and its control I fear that publications—owing to limitations of space (because of expense and editorial peculiarities) semantic difficulties and failure to appreciate the other man's approach—*have not been bringing us closer together* in an understanding of our mutual problems To me this conference has been of tremendous value I frankly have not been able to understand certain publications This conference has made it possible for me not only better to appreciate the methods and technics of our foreign colleagues but also to understand better my own countrymen and even my own close personal associates I do not think this could have developed in the course simply of reading I strongly feel that conferences such as this fulfill a great need in scientific communication and understanding I wish to acknowledge on the part of all of us our debt to the USPHS* and its advisory councils for the wisdom of recognizing the need for another method of communication than the written word I firmly believe there is no better means of bringing different scientific disciplines together and of developing appreciation of each others scientific problems and aspirations than a conference such as this one Yet there is danger in having conferences too frequently Possibly an indication for a conference is the existence of published differences of opinion and results that are growing further apart with time

I hope my colleagues on the organizing committee will forgive me for stating that in my opinion there is some danger in having too full a program The agenda was too big and the time too short Each chairman had to close the session with participants still anxious to engage in further stimulating discussion Despite the fullness of the program, I am sure it is the unanimous opinion of all participants and observers that much progress was made in understanding each others problems approaches and future plans Much new research will be generated as a result of this conference Much research under way will have its direction changed or will benefit by the introduction of new technics or the dropping of technics now shown to be inadequate or limited in effectiveness

As Professor Wintrobe stated in his opening address the participants came with finished manuscripts All of us could write a better manuscript now but these were the rules to expedite publication and everyone has agreed to abide by them I further believe this rule to be a good one for a conference and that it will be helpful to readers of the monograph to follow the development of ideas by reading the prepared manuscript and the discussion which follows all unadulterated I do not envy Dr Stohlman's task of editing the manuscripts but his assumption of the task assures the prompt publication which makes the work available to the scientific public at large

Lastly on behalf of all of us I wish to thank Professor Wintrobe his wife and associates for their efforts in making all of the local arrangements from arrival to departure The excellence of these arrangements added immensely to the success of the conference

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* U S Public Health Service

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